REMARKS

The Examiner rejected Claims 1-8, 13-15, 51 and 52 under 35 U.S.C. 103(a) as allegedly unpatentable over Arstila et al. (Science, 286:958-961, 1999), Wagner et al. (PNAS USA, 95:14447-14452, 1998), Lebed et al. (J. Biomol. Struct. Dynam., 18:813-823, 2001), Kamb et al. (US Pat. 6,060,240), Cho et al. (Appln. Env. Microbiol., vol. 68, pp. 1425-1430), and Piechocki et al. (J. Immunol. Meth., 259:33-42, 2002). Applicants respectfully disagree with this rejection.

As part of this rejection, the Examiner cites Artila and Wagner as teaching the measurement of T-cell diversity. Applicants respectfully submit that neither of these references actually quantify diversity as recited in the present claims. Arstila et al. do not enumerate TCR V beta. Instead the method originates a distribution of frequencies of CDR3 lengths, called "spectratyping." The method allows detection of shifts in the distributions which are commonly caused by oligoclonal expansions of T cells. Thus, because each CDR3 length signal is contributed by many different clones, changes in the number and diversity of T cells distributed equally by many CDR3 lengths, are left undetected. These shortcoming in such spectratyping methods are described in Turner et al. (at Tab A), which recites:

> Spectratyping allows rapid evaluation of antigen-specific TCRs within large T cell pools, but has the dual limitations that the amplification of cDNA from bulk T lymphocyte populations may introduce an element of bias and that the variation within a particular CDR3β length is not measured. (page 549, 2nd column).

In regard to Wagner et al., this reference determines frequencies of given Vbeta –Jbeta containing sequences. Because each Vbeta Jbeta combination comprehends many different clones, the method of Wagner et al does not enumerate TCRV beta diversity. Moreover, the art (Tab B, Dare et al.) has commented on the limitations of Wanger et al., stating:

> Wagner et al. (1998) quantified only about 50% of clones initially selected. The clones that were not detected were almost certainly the smallest clones, and if their size is unknown, the size of the repertoire will also be uncertain. (page 9, 1st column).

In light of the lack of quantification in Arstila et al. and Wagner et al., Applicants respectfully submit that this rejection should be withdrawn. Nonetheless, in order to further the prosecution of the present application, without acquiescing to the Examiner's rejection, while reserving the right to prosecute the original or similar claims in the future, Applicants have amended the claims. In particular, Claim 1 has been amended with the limitations from Claims 2

and 6 (which have been cancelled) to recite:

> wherein said nucleic acid molecules within said population are attached to a solid substrate, and wherein said solid substrate comprises a plurality of discrete regions, wherein each of said discrete regions comprises a different random nucleic acid molecule or an unselected express sequence tag,

Claim 1 has also been amended, in part c), to recite determining the frequency of hybridization "in each of said discrete regions."

Applicants respectfully submit that the combined art, besides not providing actual quantification, does not teach determining the frequency of hybridization (i.e., the number of hits) and instead simply measures the intensity of fluorescence (see, e.g., Cho et al. and Piechocki et al.). Applicants submit that the present application was the first to show that T-cell diversity could be enumerated by this method (empirically determined), and further submit that this method provides a direct quantification as a result – where the cited prior art only provides estimates. As such, Applicants request that this rejection be withdrawn and the claims allowed.

CONCLUSION

Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicants encourage the Examiner to call the undersigned at 608-662-1277.

Dated: December 14, 2010

/ Jason R. Bond /

Jason R. Bond
Registration No. 45,439

CASIMIR JONES S.C.
2275 Deming Way
Suite 310
Middleton, WI 53562
608.662.1277

TAB A

Turner et al., Immunity, 18:549-559, 2003

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Analysis of Clonotype Distribution and Persistence for an Influenza Virus-Specific CD8$^+$ T Cell Response

Stephen J. Turner, Gabriela Diaz, Richard Cross, and Peter C. Doherty*
Department of Immunology
St. Jude Children's Research Hospital
332 North Lauderdale
Memphis, Tennessee 38105

Summary

The spectrum of TCR Vβ usage is compared for primary and recall CD8$^+$D^{b}PA$_{224}^+$ T cell responses in mice with influenza pneumonia. Single-cell RT-PCR established that the same clonotypes were present in the lymphoid tissue and in the virus-infected lung. Longitudinal analysis indicated that the memory TCR repertoire reflects the primary response, with no decrease in diversity prior to (or after) secondary challenge. The re-engagement of memory T cells looked to be stochastic in this localized, transient infection. Analysis of clonotypes from the blood, spleen, regional lymph nodes, bone marrow, lung, and liver over a 200 day interval showed no evidence of selective localization or loss. The long-term distribution of memory T cells seemed to be essentially random.

Introduction

Respiratory infection of C57Bl/6J (B6) mice with the A/HKx31 (H3N2) influenza A virus causes severe pneumonia but relatively little damage to other tissues (Allan et al., 1990; Doherty et al., 1992; Doherty and Christensen, 2000). This localized pathology reflects that the production of new virus requires cleavage of the surface hemagglutinin (H) molecule by an enzyme restricted in distribution to the superficial epithelial layer of the respiratory tract (Rott et al., 1995; Walker et al., 1992). Plasma viremia is not detected, and viral proteins are thought to be carried to the lymph nodes and spleen in nonproductively infected dendritic cells (Hamilton-Easton and Eichelberger, 1995; McWilliam et al., 1997; Usherwood et al., 1999). The consequence is that, unlike the situation for systemic pathogens like lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* (Busch et al., 1998b; Murali-Krishna et al., 1998), the numbers of antigen-presenting cells (APCs) are likely to be relatively low in the responding lymphoid tissue. While CD8$^+$ T cells recovered by bronchoaveolar lavage (BAL) of mice with primary influenza pneumonia show potent cytotoxic T lymphocyte (CTL) activity, such effector function is minimal in the draining mediastinal lymph nodes (MLN) and spleen (Allan et al., 1990). The influenza model thus allows us to ask whether the CTL precursors (CTLp) that are recruited to the high virus environment of the infected respiratory tract following intranasal (i.n.) challenge are representative of the clonal spectrum that expands in the low virus milieu of the lymphoid tissue. Are these relationships comparable for primary and secondary responses? Do the tissue distribution profiles of memory T cells change with time?

Clonality is defined by the T cell receptor (TCR). The specificity of the TCR αβ heterodimer is determined by the variable (V), diversity (D), and joining (J) elements that first rearrange and are then transcribed together with the constant (C) region (Davis and Chien, 1999). Most of the variability is a function of the complementarity determining regions (CDR) 1, 2, and 3, particularly CDR3. Different VDJ combinations, imprecise joining, and the introduction of template-independent nucleotides lead to a great diversity of CDR3 types, allowing antigen-specific T cell responses to be characterized by restrictions in TCR V-region usage and/or CDR3 loop length (Aebischer et al., 1990; Cose et al., 1995; Kelly et al., 1993; Pantaleo et al., 1994; Yanagi et al., 1990). Functional and crystallographic analysis of the interaction between the MHC class I-peptide complex and the TCR indicates that peptide recognition is predominantly mediated via CDR3β (Garboczi et al., 1996; Garcia et al., 1996; Jorgensen et al., 1992; Lone et al., 1994; Turner et al., 1997; Wang et al., 1998).

Sequential analysis of TCR utilization in antigen-specific primary, memory, and recall CD8$^+$ T cell responses has depended on several types of approaches. Maryanski et al. (1996) dissected the evolution of an alloantigen-specific repertoire by sequencing TCRs from single cells selected on the basis of V-region usage and activation marker expression. LCMV-specific T cell populations were sorted subsequent to tetramer staining, then analyzed by RT-PCR to determine Jβ usage and the length of the CDR3β regions (Blattman et al., 2000; Lin and Welsh, 1998; Sourdive et al., 1998). This "spectratyping" protocol depends on the fact that the CDR3β lengths of naive T cells follow a Gaussian distribution (Bousso et al., 1998; Lin and Welsh, 1998; Pannetier et al., 1993; Sourdive et al., 1998), while antigen-induced selection results in the expansion of TCRs with skewed CDR3β profiles. Spectratyping allows rapid evaluation of antigen-specific TCRs within large T cell pools, but has the dual limitations that the amplification of cDNA from bulk T lymphocyte populations may introduce an element of bias and that the variation within a particular CDR3β length is not measured. Despite the technical differences, the findings from both the sequencing and the spectratyping approaches indicated that the extent of TCR diversity is comparable for primary and secondary responses (Blattman et al., 2000; Lin and Welsh, 1998; Maryanski et al., 1996; Sourdive et al., 1998).

Somewhat different conclusions were reached following single-cell sequence analysis of the CD4$^+$ T cell response to pigeon cytochrome C (McHeyzer-Williams et al., 1999; McHeyzer-Williams and Davis, 1995). The antigen-specific TCR repertoire narrowed sequentially following primary exposure, through memory to secondary challenge. Similar patterns were inferred for an *L. monocytogenes*-specific, tetramer$^+$CD8$^+$ T cell response (Busch et al., 1998a), following analysis with TCRVβ-specific monoclonal antibodies (mAbs).

*Correspondence: peter.doherty@stjude.org

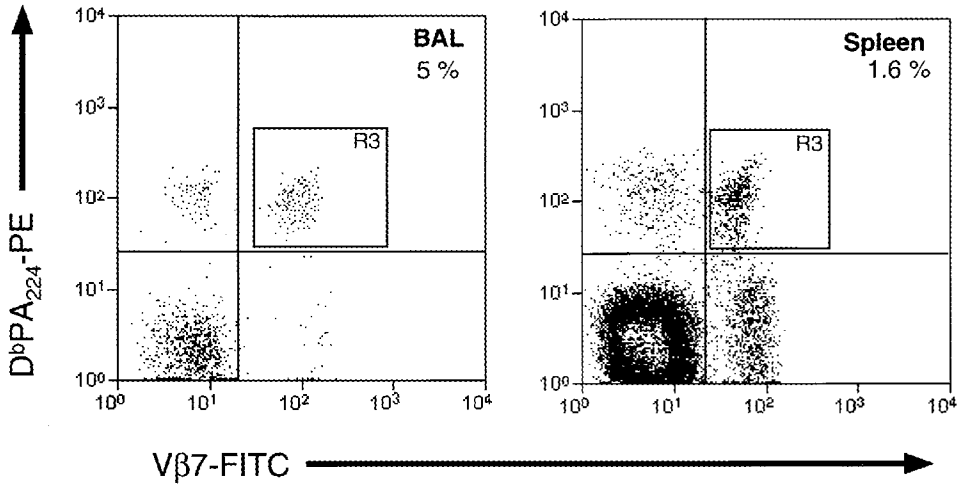


Figure 1. Typical FACS Profiles for $CD8^+V\beta7^+D^bPA_{224}^+$ T Cells

Lymphocytes were isolated from the BAL and spleens of B6 mice that had been challenged i.n. with the HKx31 (H3N2) at 8 weeks after i.p. priming with the PR8 (H1N1) virus. The lymphocytes were stained with D^bPA_{224}-PE, aCD8α-APC, and aVβ7-FITC, gated as shown, then single-cell sorted on gate R3 using a MoFlo flow cytometer. The percentage of $CD8^+D^bPA_{224}^+$ $V\beta7^+$ T cells is shown.

The present experiments analyze profiles of CDR3β usage in the host response to the influenza A virus acid polymerase ($PA_{224-233}$) peptide (SSLENFRAYV) presented by $H2D^b$ (Belz et al., 2000a, 2000b). The choice of this particular epitope was determined by the fact that a substantial proportion of these $CD8^+D^bPA_{224}$-specific T cells utilize a $V\beta7^+$ TCR (Belz et al., 2000a). Lymphocyte populations recovered from the virus-infected respiratory tract, blood, spleen, and various other tissue sites were stained for the expression of CD8α, Vβ7, and TCRs that bind the D^bPA_{224} tetramer, then sorted in the single-cell deposition unit of a flow cytometer. The naive repertoire was analyzed for $CD8^+V\beta7^+CD44^{low}$ T cells from uninfected mice that were processed in the same way. Individual CDR3β transcripts were expanded by RT-PCR (McHeyzer-Williams et al., 1999) and sequenced. This is, to our knowledge, the first analysis correlating clonal availability in the lymphoid tissue (spleen) with profiles of recruitment to a site of inflammatory pathology (lung). It is also, to our knowledge, the first account of TCR repertoire usage for the primary, memory, and recall responses in a localized infection, and the first time that the clonal distribution of memory T cells to a variety of somatic tissues has been investigated.

Results

Quantitation of Primary and Secondary $CD8^+V\beta7^+$ D^bPA_{224}-Specific Responses

The relative prevalence of $CD8^+V\beta7^+$ $D^bPA_{224}^+$ T cells (Figure 1) is shown (Table 1) for the BAL and spleen compartments of six individuals that were used to analyze the primary and secondary response (Figure 2 and Table 2). The B6 mice were either immunologically naive (#1–3, Table 1) or had been primed (#4–6, Table 1) intraperitoneally (i.p.) with the A/PR/8/34 (PR8, H1N1) virus at least 1 month prior to i.n. challenge with the HKx31 (H3N2) virus (Allan et al., 1990). These two influenza variants share the same internal components, but their surface H and neuraminidase (N) glycoproteins differ to the extent that there is no crossneutralization by antibody (Flynn et al., 1998; Kilbourne, 1969). The profile of virus replication, and thus the antigen load, is generally equivalent for the first 5 days following primary or secondary challenge with the HKx31 virus (Flynn et al., 1999).

The recall response to HKx31 was associated with (on average) 8× higher $CD8^+D^bPA_{224}^+$ T cell numbers in the BAL and 10× more in the spleen than at an equivalent stage following primary exposure, confirming that there is indeed clonal expansion following secondary i.n. challenge (Flynn et al., 1999; Marshall et al., 2001). Furthermore, the $CD8^+D^bPA_{224}^+$ cell counts were 7× (primary) and 8× (secondary) greater in the spleen than the BAL, providing ample scope for the operation of some selective process in the recruitment of immune T cells to the infected lung. The BAL results in Table 1 might indeed be interpreted as indicating that there is a greater tendency for the $V\beta7^+$ set of D^bPA_{224}-specific T cells to localize to the site of inflammatory pathology following secondary challenge. We thus repeated the FACS phenotype analysis for more individuals (Figure 1 and data not shown) and found that the percent $V\beta7^+$ in the $CD8^+D^bPA_{224}^+$ set recovered from the BAL was 51.8 ± 12.8 and 48.0 ± 14.8 for the primary and secondary responses, respectively. The comparable values for the spleen were 43.5 ± 17.7 and 52.5 ± 10.9. Clearly, these results do not suggest that there is any pattern of differential, TCR-related recruitment to the infected respiratory tract.

Characteristics of the Responding Clonotypes

The extent of repertoire diversity for the $CD8^+V\beta7^+D^bPA_{224}^+$ set was determined by sorting single cells (Figure 1), then amplifying and sequencing the CDR3β region. No analysis was made of TCR α chain diversity and, though the term "clonotype" is used in this discussion, the num-

Table 1. Quantitative Comparison of Primary and Secondary CD8+ Vβ7 D^bPA_{224}+ Responses

	BAL		Spleen	
	No.$CD8^+D^bPA_{224}$	%$V\beta7^+D^bPA_{224}$	No. $CD8^+D^bPA_{224}^+$	%$V\beta7^+D^bPA_{224}$
Primary				
Mouse #1	7.5×10^4	48%	4.7×10^5	42%
Mouse #2	10.3×10^4	33%	8.4×10^5	64%
Mouse #3	5.5×10^4	35%	3.4×10^5	43%
Secondary				
Mouse #4	6.3×10^5	58%	5.2×10^6	45%
Mouse #5	5.5×10^5	56%	4.8×10^6	54%
Mouse #6	9.7×10^5	77%	7.3×10^6	68%

Naive or primed (PR8 8 weeks previously) B6 mice were infected i.n. with $10^{6.8}$ EID_{50} of HKx31. Lymphocytes were isolated from BAL and spleen, enriched for $CD8^+$ T cells by magnetic depletion, 9 (primary) or 7 (secondary) days after infection. The T cells were then stained with the D^bPA_{224}-PE tetramer and directly conjugated anti-Vβ7-FITC and anti-CD8α-APC and analyzed on a FACSCalibur prior to sorting.

bers of such "clones" that we define here by determing CDR3β sequences is likely to underestimate the extent of diversity. The decision was made to characterize a very large number of CDR3β signatures rather than to focus on a much more limited analysis of TCRαβ phenotypes. The present measure of TCR uniqueness is thus in the amino acid (aa) sequence, and the relationships between different TCRVβs determined by patterns of CDR3β length and Jβ usage. These parameters were compared initially for naive $CD8^+V\beta7^+CD44^{low}$ T cells (data not shown) and for $CD8^+V\beta7^+D^bPA_{224}^+$ responders recovered from a variety of sampling sites following primary or secondary challenge with the HKx31 virus. We discuss the complete data set (Figures 2–5 and unpublished data) from the aspect of CDR3β region diversity here, then describe the various experiments later.

Single-cell analysis (129 sequences from three individuals) of the $CD8^+V\beta7^+CD44^{low}$ set recovered from the spleens of normal mice (data not shown) gave the expected Gaussian-like distribution (modal value 9 aa) in CDR3β length for the preimmune population (Lin and Welsh, 1998; Pannetier et al., 1993; Sourdive et al., 1998). All Jβ-gene elements were represented in the naive set, with a modest emphasis on Jβ2 (data not shown). In total, we sequenced the CDR3β regions of 1513 $CD8^+V\beta7^+D^bPA_{224}$-specific T cells from 11 mice. The modal aa length of the 241 individual sequences recovered was 6 (with 95% being from 5–7), and 72% had a glutamic acid at position 98 or 99. The spectrum of Jβ usage varied to some extent between mice, but Jβ2S6 (41%), Jβ1S1 (39%), Jβ1S5 (7%), and Jβ1S4 (4%) were the most prevalent overall. In all, 99% of the sequences expressing Jβ2S6 and 82% of those with Jβ1S1 had a glutamic acid in the CDR3β region. There were no "public" TCR signatures. While 27/241 (11%) were found in more than one mouse, none was detected in >3 of the 11 sampled. Of these "repeat" clonotypes, 23/27 were Jβ2S6 or Jβ1S1 and had the characteristic glutamine signature at position 98 or 99. Thus, there is, as would be expected, a clear pattern of selection from the naive repertoire. Though the response are to some extent "individualized," the interaction between the $V\beta7^+$ TCR and D^bPA_{224} epitope does lead to a profile of a shortened CDR3 length and skewed Jβ usage and shows a pattern of preference for a glutamic acid in the CDR3β region.

Localization of Clonotypes in Spleen and Lung following Respiratory Challenge

Comparison of the TCR sequences recovered from the BAL and spleen of individual mice demonstrated that the repertoire was essentially similar for the $CD8^+V\beta7^+D^bPA_{224}^+$ T cells that were present in these two sites (Figure 2). Sequences that were prominent (i.e., >10% of the total isolated) in the BAL were also at high frequency in the spleen (Figure 2), with the same correlation also being found for those that were less prevalent (<5%). The results for the six mice that were analyzed (Table 1) are summarized in Table 2. The pattern for the primary response was that >70% of the sequences were detected in both the spleen and the lung, with this concordance being slightly higher (>80%, Table 2) following secondary challenge. The probability that a particular T cell will localize to a site of virus induced pathology can thus be predicted from the clonal frequency in the spleen.

The sequences found exclusively in either the BAL or spleen were generally low prevalence (Figure 2), and the fact they were not identified in a site may reflect limitations in sampling. The sequences found exclusively in the BAL (4%–21%) could have been derived from clones originating in the regional MLN (Flynn et al., 1998; Tripp et al., 1995), though any large expansions in this site might also be expected to disseminate to the spleen. The 7- to 8-fold difference in the $CD8^+V\beta7^+D^bPA_{224}^+$ T cell counts from the BAL and spleen (Table 1) provides an obvious explanation for the fact that some clones (6%–14%) did not make it to the lung. What is very clear from these results is that there is no pattern of preferential recruitment of a subset of clones to the site of pathology based on the use of particular TCRβ chains.

The detailed analysis of TCR sequences (Figure 2) also confirmed the conclusion reached from the examination of CDR3β length and Jβ utilization profiles: that the primary and secondary immune repertoires are likely to be equally diverse. The percentage of clonotypes relative to the number of sequences analyzed (per mouse) ranged from 27%–30% in the primary response compared with 22%–30% following secondary challenge, with the total number of clones being 60 and 56, respectively (Table 2). This range corresponds broadly to similar estimates derived from the examination of

A

CDR3 Region	Jβ	Length	Frequency BAL	Frequency Spleen
SLDRGEV	1S1	7	**6/39**	**8/37**
SLGGSA	1S5	6	**6/39**	**3/37**
SLSPERL	1S4	7	**4/39**	**2/37**
SWDRAEV	1S1	7	**3/39**	**4/37**
SPDRGQG	1S1	7	3/39	0/37
SFGGEV	1S1	6	**3/39**	**1/37**
SGGDEQ	2S6	6	3/39	0/37
SPDRAEQ	2S6	7	**2/39**	**4/37**
SRGDEQ	2S6	6	2/39	0/37
SRGGEV	1S1	6	1/39	0/37
SWGDEV	1S1	6	1/39	0/37
SFGERL	1S4	6	1/39	0/37
SYGGAP	1S5	6	**1/39**	**2/37**
SPGQAP	1S5	6	**1/39**	**3/37**
SFGAEQ	2S1	6	1/39	0/37
SAYEQ	2S6	5	**1/39**	**1/37**
SWGGEQ	2S6	6	0/39	3/37
SLSWDREREV	1S1	10	0/39	2/37
SLDRAEV	1S1	7	0/39	2/37
SQGARV	1S4	6	0/39	1/37
LTPFNYAEQ	2S1	9	0/39	1/37

B

CDR3 Region	Jβ	Length	Frequency BAL	Frequency Spleen
SSGERL	1S4	6	**5/29**	**9/43**
SLGGYEQ	2S6	7	**4/29**	**4/43**
SPSGQL	2S2	6	**3/29**	**7/43**
SLDRGEV	1S1	7	**3/29**	**3/43**
SWGAEQ	2S6	6	**3/29**	**3/43**
SFGGEV	1S1	6	**2/29**	**5/43**
SPDRGEQ	2S6	7	**2/29**	**2/43**
SFGERL	1S4	6	**2/29**	**1/43**
SLGGEV	1S1	6	1/29	0/43
SLDRGGL	1S4	7	**1/29**	**1/43**
SLGDEQ	2S6	6	**1/29**	**1/43**
STWTDEQ	2S6	7	1/29	0/43
SSEGDEQ	2S6	7	1/29	0/43
SLGAEQ	2S1	6	0/29	2/43
EDRGANTGQL	2S2	10	0/29	1/43
SFGERG	2S6	6	0/29	4/43

Figure 2. Comparison of Sequences from the Spleen and BAL after Primary or Secondary Challenge

The deduced amino acid sequences of the CDR3β region are shown for single $CD8^+V\beta7^+$ $D^bPA_{224}^+$ T cells from two mice sampled after primary (A) or secondary (B) challenge with the HKx31 virus. The sequences that were found in both the BAL and spleen are highlighted in bold. The TCR Vβ7 sequence was deduced from comparison with the published TCR Vβ7 sequence (Arden et al., 1995; Iwamoto et al., 1987; Saito et al., 1984). The Jβ regions were assigned according to Gascoigne et al. (1984) and Malissen et al. (1984). The data shown here are for #1 and #4 identified in Tables 1 and 2.

alloantigen-specific $CD8^+$ T cell repertoires (Maryanski et al., 1996, 2001; Walker et al., 1996). Even so, the present comparisons were all made between different mice. What is the situation when the same individual is analyzed sequentially?

Analysis within Individuals

Determining T cell repertoire profiles for the primary, memory, and recall response within the same mouse is subject to obvious technical constraints that place a limit on sample size. The strategy used was to characterize the $CD8^+V\beta7^+D^bPA_{224}^+$ set in the peripheral blood lymphocyte (PBL) pool obtained at the acute phase (d7) of the response following i.p. exposure to the PR8 virus, and at an early stage of immune memory (d21). The mice were then rested for 12 weeks prior to i.n. challenge with the HKx31 virus, when the BAL and spleen populations were sampled as before. The sequence data for 2/3 mice that were analyzed in this way are presented in Figure 3.

The concordance for sequences present on both d7 and d21 was approximately 86% (range 85–87) for the three mice (Figures 3A and 3B, and data not shown). Most (if not all given the limited sample size) of the clones expanded during the antigen driven phase (d7) thus seem to persist into the initial stage (d21) of immune memory. More specifically, comparison of the d7 and d21 repertoires in #7 (Figure 3A) demonstrated that 4/5 of the prominent (>10%) clonotypes found early were also present later. For example, the sequence SFGGEQYFG (#7) was detected at a frequency of 6/29 and 4/37 on d7 and d21, respectively. In #8 (Figure 3B), SFGGEVFFG was found in 4/20 on d7 and 13/47 on d21. A similar pattern was recognized for 4/5 of the prominent clonotypes identified in #9 (data not shown). The establishment of memory may thus be considered to reflect the size of the clonal burst following the initial encounter with antigen (Hou et al., 1994).

Comparison of the number of different clonotypes within the primary, memory, and secondary repertoires supported the conclusion reached from the analysis of CDR3β and Jβ usage (Figure 2 and data not shown) that the overall breadth of diversity does not change through these sequential phases of the host response. The numbers of distinct clonotypes found on d7, on d21, and after the d90 challenge were: 13, 16, and 15 (#7, Figure 3A); 11, 15, and 15 (#8, Figure 3B); and 10, 12, and 17 (#9, data not shown). The diversity between each repertoire can, in fact, be considered to reflect three distinct patterns. The first was that some sequences were found only after secondary challenge (Figures 3A and 3B and data not shown). With the exception of one signature detected in 7/66 sequences from #8, (Figure 3B, SLSGYEQYFG), all the "new" clones were found at low prevalence (<5%). The obvious possibilities are that these originated from low-frequency clonotypes missed in the limited analysis of the PBL on d7 and d21, or were derived from the expansion of naive T cells (Turner et al., 2001) that had either failed to encounter an APC during the initial infection or had emerged from the thymus over the subsequent 90 days. The second pattern was that sequences found on both d7 and d21 were not detected again following the HKx31 challenge. Again, these were usually low frequency (<5%) clonotypes, though the TGATEVFFG signature (Figure 3B) was present at >10% in both the primary and memory repertoires but was lost in the recall response. Last, some sequences were detected at all three time points (bold type, Figure 3). The percentage of clonotypes isolated from the primary repertoire (d7 or d21) that were also found following secondary challenge was 72% (#7), 44% (#8), and 57% (#9), respectively (Figures 3A and 3B and data not shown). These results indicate that the majority of the secondary repertoire is selected from the circulating pool of $CD8^+$ memory T cells, especially when we

Table 2. Patterns of Clonotype Distribution in the BAL and Spleen

		% Sequence Observed in Site		
	No. Clonotypes	BAL/Spleen	BAL Only	Spleen Only
Primary				
Mouse #1	21 (n = 76)	72%	16%	12%
Mouse #2	16 (n = 52)	73%	21%	6%
Mouse #3	23 (n = 73)	70%	16%	14%
Secondary				
Mouse #4	16 (n = 66)	86%	9%	5%
Mouse #5	16 (n = 72)	86%	4%	10%
Mouse #6	24 (n = 90)	81%	11%	8%

The results are for the mice identified in Table 1. Detailed sequence data for mouse #1 and mouse #4 are presented in Figure 2.

consider the effect of limited sample size (see following section).

Though some sequences were present at equivalent frequency in the primary, memory, and secondary repertoires, the correlation did not always hold. While SWAVEQYFG was, for example, prominent on d7 (14%) and d21 (11%), this TCR was used by only 3% of clones following secondary challenge (Figure 3A). Conversely, SLSGEQYFG (Figure 3A) was found in only 1/66 clones sampled on d7 or 21, compared with 11% in the recall response. The overall impression is that, while the primary repertoire selected during the acute phase of the response is stable into memory, the involvement of any particular memory T cell following secondary challenge will be more random (Figures 3A and 3B and unpublished data). Further clonal expansion presumably depends on the chance encounter between antigen-specific $CD8^+$ T cell and APC in an appropriate lymphoid microenvironment, an event that will be influenced in turn by the antigen load.

Distribution of Long-Term Memory Populations to Different Anatomical Sites

While the majority of sequences found in the secondary repertoire had also been detected following primary challenge, some (generally low-frequency) clonotypes were unique to the primary/memory (PBL) and/or secondary (BAL/spleen) $CD8^+V\beta7^+D^bPA_{224}^+$ populations (Figures 3A and 3B). This could be a function of the limited sample size, the selective distribution of memory T cells to extravascular sites (Huleatt et al., 2001) or the involvement of new clones (Turner et al., 2001). How faithfully does the PBL population reflect the total $CD8^+$ memory T cell pool? A detailed temporal and anatomical analysis was thus made of the $CD8^+V\beta7^+D^bPA_{224}^+$ T cell distribution profiles for two secondarily challenged (HKx31→PR8) mice (#10 and #11, Figures 4 and 5, respectively). The focus on the recall response reflects that the numbers of memory T cells recovered from (in particular) nonlymphoid tissues would have been too small following primary exposure to the HKx31 virus. The prevalence of $CD8^+V\beta7^+D^bPA_{224}^+$ T cells in the different compartments was consistent between the different compartments (Figure 4A) with the percentage ranging from 40%–58% in the blood, spleen, MLN, bone marrow (BM), liver, and lung (Figure 4A, parentheses). Clonotypes were isolated from the blood on days 8, 35, 100, and 200 (Figures 4B and 5A) and from the spleen, MLN, BM, lung, and liver on d200 (Figures 4C and 5B).

The dominant (>10%) sequences identified in the PBL pool soon after secondary challenge (d8) also tended to be at high frequency on d35, d100, and d200, supporting the earlier conclusion that memory reflects the antigen-driven phase of the response and is stable over time (Figures 3, 4B, and 5A). Such prominent clonal signatures represented 78% and 60% of total sequences isolated from the PBL compartment of mouse #10 and #11, respectively (Figures 4B and 5A). Even so, these distribution profiles were not invariant. While SLDREGV (Figure 5A) was detected at >10% on d8, d100, and d200, it was absent on d35. Conversely, SLAHRDTNSDY was generally present at low frequency but was found in 8/46 sequences from the d100 PBL population. Also, though this single-cell PCR approach did not allow us to look closely at other characteristics of the memory T cell pool, no significant difference in the extent of tetramer staining was detected at the T cell population level over the 200 day sampling period (on days 8, 35, 100, and 200: mean fluorescence intensity = 81, 78, 75, 78 for #10; and 68, 54, 77, 78 for #11). Though this is a very crude measure of TCR avidity, the lack of variability again indicates that long-term memory in the absence of antigen is characterized by stability rather than continuing positive or negative selection.

The earlier analysis within individuals (Figures 3A and 3B) suggested that some of the apparent secondary response might reflect the involvement of naive T cell clones (Turner et al., 2001). Another explanation could be, however, the mobilization of memory T cells dispersed in tissue sites but not normally present in the blood. This might be a random process, or reflect selective localization to sites of prior antigen exposure and/or pathology (MLN and lung in this influenza model) (Huleatt et al., 2001). The two mice that had been analyzed from d8 to d200 (Figures 4B and 5A) were thus exsanguinated, then perfused, prior to sampling the MLN, lung, spleen, BM, and liver compartments. In both cases, the clones that had been prominent throughout in the PBL were also present at high frequency in the various tissues. There was no obvious pattern of selective recovery from the lung or MLN, the sites that are most exposed following influenza virus infection.

The PBL profiles (Figures 4B and 5A) were not, however, totally predictive of the patterns found in other sampling sites (Figures 4C and 5B). In #10, 55% of the

A

			Primary		Secondary	
CDR3	Jβ	Length	D7	D21	BAL	Spleen
SFGGEQ	2S6	6	**6/29**	**4/37**	**2/43**	**1/47**
SFGDEQ	2S6	6	**4/29**	**4/37**	**3/43**	**2/47**
SWAVEQ	2S6	6	**4/29**	**4/37**	**3/43**	**0/47**
SLGGEV	1S1	6	**3/29**	**0/37**	**8/43**	**6/47**
SFGAEQ	2S1	6	**3/29**	**5/37**	**4/43**	**10/47**
SLGGEQ	2S6	6	**2/29**	**6/37**	**1/43**	**2/47**
SSGGGV	1S1	6	**2/29**	**1/37**	**1/43**	**0/47**
SLAGYEQ	2S6	7	**2/29**	**1/37**	**8/43**	**6/47**
SLDRGEV	1S1	7	**2/29**	**2/37**	**1/43**	**1/47**
SLGREV	1S1	6	**1/29**	**2/37**	**0/43**	**1/47**
SPDRGRV	1S1	7	**1/29**	**1/37**	**5/43**	**7/47**
SSTGEV	1S1	6	1/29	2/37	0/43	0/47
SGQGGETL	2S3	8	1/29	0/37	0/43	0/47
SSYTEV	1S1	6	0/29	1/37	0/43	0/47
QQGGNTL	1S2	6	**0/29**	**1/37**	**0/43**	**3/47**
STGLEV	1S1	6	0/29	1/37	0/43	0/47
SLSGEQ	2S6	6	**0/29**	**1/37**	**4/43**	**7/47**
SGGTEV	1S1	6	0/29	1/37	0/43	0/47
SAPDEQ	2S6	6	0/29	0/37	1/43	0/47
SYGEGQ	2S6	6	0/29	0/37	2/43	1/47

B

			Primary		Secondary	
CDR3	Jβ	Length	D7	D21	BAL	Spleen
SFGGEV	1S1	6	**4/20**	**13/47**	**4/29**	**4/37**
TGATEV	1S1	6	**4/20**	**9/47**	**0/29**	**0/37**
SSGQAP	1S5	6	**2/20**	**4/47**	**5/29**	**11/37**
SFGERL	1S4	6	2/20	3/47	0/29	0/37
SWGDEQ	2S6	6	**2/20**	**2/47**	**8/29**	**5/37**
SWGKSL	2S3	6	1/20	0/47	0/29	0/37
SPDRGSL	2S3	7	**1/20**	**2/47**	**1/29**	**7/37**
SQGGEQ	2S6	6	1/20	0/47	0/29	1/37
SPDRGEV	1S1	7	**1/20**	**0/47**	**1/29**	**0/37**
SFGDEQ	2S6	6	1/20	3/47	0/29	0/37
SGLGGYEQ	2S6	8	**1/20**	**4/47**	**1/29**	**0/37**
SLGGEQ	2S6	6	0/20	1/47	0/29	0/37
SRGGEV	1S1	6	0/20	1/47	0/29	0/37
TGGGV	1S1	5	**0/20**	**1/47**	**1/29**	**0/37**
SFGAETL	1S2	7	0/20	1/47	0/29	0/37
SLGREV	1S1	6	0/20	1/47	0/29	0/37
SGETL	1S2	5	0/20	1/47	0/29	0/37
TSVEQ	2S6	5	0/20	1/47	0/29	0/37
SGGGEQ	2S6	6	0/20	0/47	2/29	1/37
SLSGYEQ	2S6	7	0/20	0/47	2/29	5/37
SFGGAP	1S5	6	0/20	0/47	2/29	0/37
QGGERL	1S4	6	0/20	0/47	1/29	0/37
SLGAEQ	2S1	6	0/20	0/47	1/29	0/37
SLAGYEQ	2S6	7	0/20	0/47	0/29	1/37
SSYEQ	2S6	5	0/20	0/47	0/29	1/37
SLGDGV	1S1	6	0/20	0/47	0/29	1/37

Figure 3. Longitudinal Analysis of Primary, Memory, and Secondary $CD8^+V\beta7^+D^bPA_{224}^+$ T Cell Repertoires within the Same Mouse

Single $CD8^+V\beta7^+D^bPA_{224}^+$ cells were isolated from the PBL on d7 and d21 after i.p. infection with the PR8 virus. These mice were challenged i.n. with the HKx31 virus 90d later, and the BAL and spleen populations were recovered after a further 6d. The sequences that were detected consistently on d7, d21, and d96 are highlighted in bold. The results are for two individual mice (#7 and 8) with a third (#9) not shown.

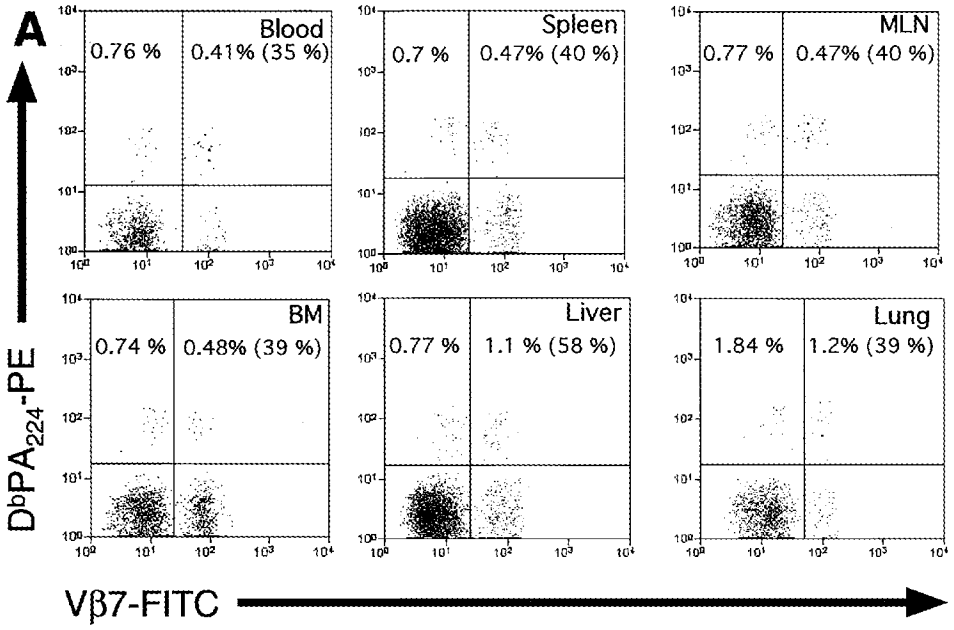
clones that were detected in the PBL were not found in the tissues, while 57% of those in the tissues had not been seen previously in the blood. The comparable values for #11 were 38% and 32%. Most of these sequences were at low frequency, with (for #10 and #11, respectively) 50% and 44% (PBL) and 61% and 100% (tissue) being recovered just once. In all, 44% (#10) and 100% (#11) of the CDR3β sequences that were restricted to tissue sites would have been missed if the analysis had been limited to the spleen. Also, though the seven sequences that were found only in the tissues and not the blood of #11 represent single isolates, 5/7 had the characteristic 6 or 7 aa CDR3β length and Jβ1S1 or Jβ2S6 profile, while 6/7 had a glutamic acid within CDR3β (see *Characteristics of the Responding Clonotypes*, above). Such "minor" clonotype cannot be discounted. Another, SLGGFEQ was found in 1/142 PBL samples, but was then detected in 6/177 from tissue sites (Figure 4). Also, SLSGEQ was present in 1/66 PBL isolates, but expanded to 11/91 found in spleen or lung after secondary challenge (Figure 3).

Thus, though conclusions can legitimately be made concerning the relative prevalence of prominent T cell clones in long-term memory, it would be unwise to be too prescriptive about patterns of possible clonotype gain or loss from analyses based solely on sampling the PBL and/or lymphoid compartment. We also looked to see whether there was any preferential localization of clonotypes to the former site of virus-induced pathology (lung) or to the responding lymph node (MLN). No such effect was apparent for the prominent (>10% in any site) or minor (<5% in any site) clonotypes. There is thus no tendency in this influenza virus model for long-term $CD8^+$ memory T cells to "home" back to the site where they were originally stimulated.

Discussion

The current dissection of $CD8^+$ T cell-mediated immunity to the D^bPA_{224} influenza A virus epitope supports the idea that the immune repertoire generated following antigen challenge is drawn from a highly selected subset of the available TCRs that enter the naive, peripheral T cell pool following thymic differentiation (Arstila et al., 1999). The same conclusion has been made repeatedly from the analysis of $CD4^+$ (McHeyzer-Williams et al., 1999) and $CD8^+$ T cell reponses to both noninfectious (Maryanski et al., 2001) and infectious (Sourdive et al., 1998; Wallace et al., 2000) antigens. Furthermore, we detected a consistent pattern of restricted CDR3β length (generally 5–7 aa) and relative prominence of a limited spectrum of Jβ regions that is the hallmark of other $CD4^+$ (McHeyzer-Williams and Davis, 1995) and $CD8^+$ T cell responses (Maryanski et al., 1996).

Influenza infections are readily controlled by the adaptive immune response. Epitopes capable of stimulating $CD8^+$ T cells have not been detected beyond 14 days after primary i.n. challenge of B6 mice with H3N2 viruses (Eichelberger et al., 1991; Hamilton-Easton and Eichelberger, 1995). The present analysis showed that CTLp that are prominent in the primary response (d7) persist into both short- and long-term memory and are generally (though not invariably) detected again after secondary challenge (d90). Furthermore, there are subtle changes in the composition of the secondary repertoire. Our results support the model that prominent memory T cell populations tend to be those that are substantially expanded during the primary response, while selection of T cells from the memory pool following secondary challenge is a stochastic event reflecting the chance association of APC and CTLp in the lymphoid tissue (Flynn et al., 1999). Random, sporadic encounters with antigen may also explain the changing clonal hierarchies that have been described by some who work with persis-



B

		Blood			
CDR3	**Jβ**	**D8**	**D35**	**D100**	**D200**
SYGGEV	1S1	**18/43**	**15/34**	**4/28**	**9/35**
SSYEQ	2S6	**9/43**	**4/34**	**6/28**	**6/35**
SPDRGEV	1S1	**5/43**	**4/34**	**8/28**	**7/35**
SSPDEQ	2S6	**5/43**	**3/34**	**3/28**	**4/35**
TSGAEQ	2S1	2/43	0/34	0/28	0/35
SLGGEV	1S1	2/43	2/34	1/28	2/35
SLWHQNTL	1S3	1/43	0/34	0/28	0/35
SPGTVNSDY	1S2	1/43	0/34	0/28	0/35
SKDVEV	1S1	0/43	2/34	0/28	1/35
SFGEAP	1S5	0/43	1/34	1/28	1/35
SFGDEQ	2S6	0/43	1/34	1/28	2/35
SLGGFEQ	2S6	0/43	1/34	0/28	0/35
SFTGEV	1S1	0/43	1/34	2/28	3/35
SLDRGERL	1S4	0/43	0/34	1/28	0/35
SDRGETL	2S3	0/43	0/34	1/28	0/35

C

CDR3	**Jβ**	**Spleen**	**MLN**	**BM**	**Lung**	**Liver**
SPDRGEV	1S1	**8/37**	**8/30**	**5/38**	**4/38**	**8/34**
SYGGEV	1S1	**7/37**	**8/30**	**9/38**	**13/38**	**7/34**
SSYEQ	2S6	**6/37**	**6/30**	**4/38**	**7/38**	**7/34**
SSPDEQ	2S6	**5/37**	**3/30**	**7/38**	**9/38**	**6/34**
SFTGEV	1S1	2/37	0/30	1/38	1/38	1/34
SFGEAP	1S5	2/37	0/30	2/38	0/38	0/34
TAGNTL	1S3	1/37	0/30	0/38	0/38	0/34
GGYTEV	1S1	1/37	0/30	0/38	0/38	0/34
SDSAETL	2S3	1/37	0/30	0/38	0/38	0/34
SFGRGNTL	1S3	1/37	0/30	0/38	0/38	0/34
SRDRGTL	2S3	1/37	0/30	1/38	0/38	0/34
RGTGIDERL	1S4	1/37	0/30	0/38	0/38	0/34
SIRISNERL	1S4	1/37	0/30	0/38	0/38	0/34
SLDRAEV	1S1	0/37	1/30	2/38	1/38	4/34
SLGGFEQ	2S6	0/37	1/30	3/38	0/38	2/34
SSNSDY	1S2	0/37	1/30	0/38	0/38	0/34
SPGGEQ	2S6	0/37	1/30	2/38	0/38	0/34
SKDVEV	1S1	0/37	1/30	2/38	0/38	1/34
SLGGEQ	2S6	0/37	0/30	0/38	1/38	0/34
SLGGEV	1S1	0/37	0/30	0/38	1/38	0/34
SSPERL	1S4	0/37	0/30	0/38	1/38	0/34

Figure 4. Long-Term Analysis of a Memory T Cell Repertoire

Single CD8+Vβ7+DbPA224+ cells were recovered from the PBL on d8, d35, d100, and d200 after HKx31 challenge of a PR8-primed mouse (#10). On d200, single CD8+Vβ7+DbPA224+ cells were also isolated from the spleen, MLN, bone marrow, liver, and lung. Flow cytometric profiles of CD8+Vβ7+DbPA224+ T cells detected from the various organs are shown (A) with the percentage of DbPA224+ T cells shown for upper left and upper right quadrants. The percentage of DbPA224+ expressing Vb7 is shown in parentheses. Those sequences that were detected in the blood (B) and at least one other site (C) are highlighted in bold.

A

CDR3	Jβ	Blood D8	D35	D100	D200
SPDRGRV	1S1	**10/44**	**15/41**	**3/46**	**13/36**
SLGAEQ	2S1	**7/44**	**4/41**	**3/46**	**4/36**
SFGGEV	1S1	**5/44**	**6/41**	**8/46**	**6/36**
SLDRGEV	1S1	**5/44**	**0/41**	**8/46**	**4/36**
SSGAEV	1S1	3/44	2/41	1/46	0/36
STGEAP	1S5	3/44	0/41	0/46	0/36
SFGGEQ	2S6	3/44	0/41	0/46	0/36
SSGKAP	1S5	3/44	3/41	0/46	2/36
SLGGEV	1S1	2/44	0/41	0/46	0/36
SSYEQ	2S6	1/44	0/41	1/46	0/36
SPSGWGDTGQL	2S2	1/44	0/41	1/46	0/36
SLAHRDTNSDY	1S2	1/44	4/41	8/46	0/36
SFGGAP	1S5	0/44	2/41	1/46	0/36
SFGDEQ	2S6	0/44	2/41	1/46	2/36
SEGGEQ	2S6	0/44	1/41	0/46	0/36
SLTPETL	2S3	0/44	1/41	3/46	0/36
SWDRGEV	1S1	0/44	1/41	0/46	0/36
SLGDEQ	2S6	0/44	0/41	2/46	0/36
SLGQAP	1S5	0/44	0/41	2/46	0/36
SSGGEQ	2S6	0/44	0/41	2/46	0/36
SLTGEV	1S1	0/44	0/41	1/46	0/36
SSYTEV	1S1	0/44	0/41	1/46	3/36
TQGGAP	1S5	0/44	0/41	0/46	1/36
TLDRGNL	1S6	0/44	0/41	0/46	1/36

B

CDR3	Jβ	Spleen	MLN	BM	Lung	Liver
SPDRGRV	1S1	**6/32**	**11/36**	**12/46**	**7/32**	**9/44**
SFGDEQ	2S6	**5/32**	**4/36**	**5/46**	**4/32**	**4/44**
SFGGEV	1S1	**4/32**	**2/36**	**3/46**	**4/32**	**4/44**
SSGKAP	1S5	**4/32**	**1/36**	**3/46**	**1/32**	**1/44**
SLGAEQ	2S1	**3/32**	**8/36**	**7/46**	**6/32**	**11/44**
SLDRGEV	1S1	**2/32**	**1/36**	**5/46**	**5/32**	**4/44**
SSYTEV	1S1	2/32	0/36	3/46	0/32	4/44
SLAHRDTNSDY	1S2	2/32	1/36	2/46	2/32	1/44
SFGGAP	1S5	2/32	1/36	0/46	0/32	0/44
TLDRGNL	1S6	1/32	0/36	0/46	0/32	0/44
STGEAP	1S5	1/32	1/36	1/46	0/32	0/44
SLGQGGYAEQ	2S1	0/32	1/36	0/46	0/32	0/44
SLGQAP	1S5	0/32	1/36	2/46	1/32	1/44
SLGGFEQ	2S6	0/32	1/36	0/46	0/32	0/44
SLTPETL	2S3	0/32	1/36	0/46	0/32	1/44
SSGAEV	1S1	0/32	1/36	0/46	0/32	0/44
SGGGEQ	2S6	0/32	1/36	0/46	0/32	0/44
SLMGITEV	1S1	0/32	0/36	1/46	0/32	0/44
SFGGEQ	2S6	0/32	0/36	1/46	0/32	2/44
SLNYEQ	2S6	0/32	0/36	1/46	0/32	0/44
SLYQSGNTL	1S3	0/32	0/36	0/46	1/32	0/44
SLDRGEQ	2S6	0/32	0/36	0/46	1/32	0/44

Figure 5. The Distribution of Memory $CD8^+$ T Cells

Single $CD8^+V\beta7^+D^bPA_{224}^+$ memory T cells were analyzed from the PBL (A) and other sites (B) of mouse #11, as described in the footnote to Figure 4.

tent infections (Annels et al., 2000; Chen et al., 2001), though others have reported evidence of considerable clonal stability in context of continued viral load (Brander et al., 1999; Islam et al., 2001).

As would be expected, there were no indications that a TCR-related selective process operates at the level of the vascular endothelium to favor the extravasation of particular D^bPA_{224}-specific CTLp into the infected lung. All $CD8^+V\beta7^+D^bPA_{224}^+$ T cells that achieved a large clone size in the spleen following primary or secondary i.n. challenge were invariably detected in the BAL, with the levels in the two sites often being fairly comparable. A few clonotypes were found only in the BAL, perhaps reflecting an origin in the MLN or other regional lymph nodes. However, given that the secondary response has the characteristics of a stochastic process, the results do not in any way favor the idea that the events in the lung are dominated by memory T cells that happened to be trafficking through the respiratory tract and bronchus-associated lymphoid tissue at the time of virus challenge. If that were the case, a much greater dissociation would be expected between the secondary response in the BAL and spleen. The relatively low prevalence (<1.0%) of $CD8^+$ memory T cells in mice primed i.p. with the PR8 virus probably mandates a requirement for further clonal expansion in the lymphoid tissue (Flynn et al., 1998). An immediate, local response may play a much greater part when large numbers are already present in the spleen (>10%) and lung (Christensen et al., 2000; Marshall et al., 2001; Stevenson et al., 1999).

Control of an extremely virulent H7N7 virus was apparent within 3d in HKx31→PR8 double-primed mice (Christensen et al., 2000; Marshall et al., 2001), while lung titers in single-primed, PR8-immune mice remained high for at least 6d after exposure to the much less pathogenic HKx31 virus (Flynn et al., 1998).

The clonotypic diversity of primary and secondary responses for individual mice correlates with other estimates of epitope-specific CD8+ T cell repertoires (Maryanski et al., 1996; Walker et al., 1996). However, while the CD8+Vβ7+DbPA224+ repertoire shows an overall pattern of restricted CDR3β length and Jβ usage, this is by no means invariant as there is considerable diversity both within and between individuals. Each mouse developed distinct, or "private" (Cibotti et al., 1994), TCR signatures following both primary and secondary challenge. No "public" TCRs were found in every mouse. The CD8+Vβ7+DbPA224+ response is certainly reminiscent of other situations in which a polyclonal repertoire is established concurrent with a prominent profile of CDR3β and Jβ restriction (Cose et al., 1995; Kelly et al., 1993; Naumov et al., 1998; Wallace et al., 2000).

Studies of the responding CD4+ and CD8+ T cell repertoires to both inert proteins and infectious agents have led to two basic interpretations of the relationship between the antigen-driven phase of clonal expansion and established memory. One idea is that the spectrum of TCRs generated by primary challenge focuses in some way to provide a functionally superior population of memory T cells. The broad alternative is that the diversity of the memory pool is a direct reflection of the primary response and is stable over time. Analysis to date of CD4+ T cell response has tended to favor the first idea (Fasso et al., 2000; Malherbe et al., 2000; McHeyzer-Williams et al., 1999), while CD8+ memory T cells seem more likely to reflect the profiles generated during primary challenge (Busch et al., 1998a; Lin and Welsh, 1998; Maryanski et al., 1996; Sourdive et al., 1998).

Analyzing primary and secondary responses in different influenza-immune mice suggested that we were not seeing the narrowing of repertoire described for some CD4+ (McHeyzer-Williams et al., 1999; McHeyzer-Williams and Davis, 1995) and CD8+ T cell (Busch et al., 1998a) responses. The extent of diversity measured at the single-cell level looked to be broadly comparable following primary or secondary challenge. Sequential sampling within individuals indicated that the primary repertoire in blood was no more diverse than the secondary repertoire recovered from the spleen and infected lung. Longitudinal analysis of secondarily challenged mice also suggested that memory is very stable, reflects the clontoypes expanded during the acute phase of infection (Hou et al., 1994), and maintains diversity over time. Also, though it is a very crude measure of TCR avidity, there was no obvious change in the level of tetramer binding to CD8+Vβ7+DbPA224+ T cell populations recovered from blood at d8 to d200 after secondary challenge.

Though substantial numbers (142, #10: 167, #11) of CD8+Vβ7+DbPA224+ T cells had been analyzed previously from the PBL, a spectrum of "new" CDR3β sequences (12/27, #10: 7/31, #11) was recovered from tissue sites in two mice sampled at d200 after secondary virus challenge. These were generally, though not always, minor (<5%) clonotypes, suggesting that the lack of prior detection was simply a function of sample size rather that a reflection of some propensity for particular clones to lodge in the extravascular compartment. Even so, these results show very clearly that any conclusion concerning the gain or loss of TCR diversity for situations where there is no likelihood of continued antigen challenge can only be made safely for the relative prevalence of high frequency clonotypes, and then only after repeated sampling.

Experimental Procedures

Mice, Viruses, Infection, and Sampling

The 6- to 8-week C57BL/6J, female mice were purchased from The Jackson Laboratories, Bar Harbor, ME, and housed under SPF conditions. Some were infected i.p. with $10^{6.5}$ 50% embryonated hen's egg infectious dose (EID_{50}) of the PR8 (H1N1) influenza A virus (Allan et al., 1990). Naive and PR8-primed (12–16 weeks previously) mice were anesthetized by i.p. injection of avertin and challenged i.n. with $10^{6.8}$ EID_{50} of the HKx31 (H3N2) virus (Kilbourne 1969; Flynn et al., 1998). Lymphocytes were isolated from lung by BAL (Allan et al., 1990) and CD8+ T cells were enriched (Hou et al., 1994) from single-cell preparations of spleen using mAbs (Pharmingen, Torrey Pines, CA) to CD4 (GK1.5) and MHC class II (TIB120) followed by anti (a)-rat and a-mouse Ig-coated magnetic beads (Dynall AS., Oslo, Norway). The five immune mice that were analyzed longitudinally were anesthetized at intervals by isofluorane inhalation and bled via the retro-orbital sinus. The blood (50-100 μl) was diluted into 2 ml of 10 U/ml of Heparin in PBS, the RBC were lysed, and the remaining PBL were stained for flow cytometry. The protocols for tissue perfusion (to remove blood) and lymphocyte recovery from the bone marrow, liver, and lung parenchyma of primed mice have been described previously (Marshall et al., 2001).

Isolation of Single CD8+ T Cells

The lymphocytes were separated using a MoFlo sorter (Cytomation, Fort Collins, CO) fitted with a "Cyclone" single cell deposition unit. Immune CD8+ T cells were identified with the DbPA224-PE tetramer (Belz et al., 2000b), while the naive set (Tabi et al., 1988) was stained with aCD44-PE (Pharmingen). After washing in PBS/0.1% BSA and staining with aCD8α-allopyhcoerythrin (APC) (Pharmingen) and aVβ7-FITC (Pharmingen), the concentration was adjusted to 20 × 10^6/ml and single CD8+Vβ7+DbPA224+ or CD8+Vβ7+CD44low T cells were sorted directly into a 96-well PCR plate (United Scientific Products, San Leandro, CA) containing 5 μl of cDNA reaction mix. As an initial control, single cells were sorted directly into medium and visualized using a phase-contrast microscope. Such analysis demonstrated 90%–100% accuracy, with no more than one cell being found in any well. Negative controls were interspersed between the samples (1 in 10), and approximately 50–80 cells were sorted per plate.

cDNA Synthesis, Nested RT-PCR, and Sequencing

The cDNA reaction mix contained Sensiscript reverse transcriptase (0.25 μl, Qiagen, Valencia, CA) with its recommended 1× cDNA buffer, 0.5 mM dNTPs (Invitrogen, Carlsbad, CA), 0.125 μg oligo $dT_{(15)}$ (Promega, Madison WI), 100 μg/ml gelatin (Roche, Indianapolis, IN), 100 μg/ml tRNA (Roche), 20 U RNAsin (Promega), and 0.1% Triton X-100 (Sigma, St. Louis, MO). After sorting, the plates were incubated at 37°C for 90 min for cDNA synthesis, then for a further 5 min at 95°C to stop the reaction, and stored at −80°C. The Vβ7+ transcripts were amplified by nested PCR, using 2 μl of cDNA for a 25 μl amplification reaction. The first round PCR was performed with 1.5 U Taq polymerase (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen), and 10 pmol of both the external sense primer Vβ7-5′ (5′-TTGCTGGAATGTGGACAGGAC-3′) and the external antisense primer Cβa (5′-CCAGAAGGTAGCAGAGACCC-3′). The PCR conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 1 min, followed by one cycle of 95°C for 1 min, 59°C for 1 min, and 72°C for 7 min. A 2 μl aliquot of the first round PCR was used as a template for the nested PCR with

the same buffers, a Vβ7 sense (5′-TACAGGGTCTCACGGAA GAAGC-3′) and an antisense primer Cβb (5′-CTTGGGTGGAGTCA CATTTCTC-3′). The PCR conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min, followed by one cycle of 95°C for 1 min, 57°C for 1 min, and 72°C for 7 min. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and resolved on a 2% agarose gel. An aliquot of the PCR product was precipitated with equal volume 4 M ammonium acetate and 2× volume of isoproponal, then sequenced with 3.2 pmol of sense Vβ7 primer on an ABI Prism 3700 sequence analyzer.

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References

Aebischer, T., Oehen, S., and Hengartner, H. (1990). Preferential usage of Vα4 and Vβ10 TCR genes by LCMV glycoprotein-specific H-2D^b-restricted cytotoxic T cells. Eur. J. Immunol. *20*, 523–531.

Allan, W., Tabi, Z., Cleary, A., and Doherty, P.C. (1990). Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting CD4+ T cells. J. Immunol. *144*, 3980–3986.

Annels, N.E., Callan, M.F., Tan, L., and Rickinson, A.B. (2000). Changing patterns of dominant TCR usage with maturation of an EBV-specific cytotoxic T cell response. J. Immunol. *165*, 4831–4841.

Arden, B., Clark, S.P., Kabelitz, D., and Mak, T.W. (1995). Mouse TCR V gene segment families. Immunogenetics *42*, 501–530.

Arstila, T.P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., and Kourilsky, P. (1999). A direct estimate of the human αβ TCR diversity. Science *286*, 958–961.

Belz, G.T., Stevenson, P.G., and Doherty, P.C. (2000a). Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. J. Immunol. *165*, 2404–2409.

Belz, G.T., Xie, W., Altman, J.D., and Doherty, P.C. (2000b). A previously unrecognized H-2D^b-restricted peptide prominent in the primary influenza A virus-specific CD8$^+$ T-cell response is much less apparent following secondary challenge. J. Virol. *74*, 3486–3493.

Blattman, J.N., Sourdive, D.J., Murali-Krishna, K., Ahmed, R., and Altman, J.D. (2000). Evolution of the T cell repertoire during primary, memory, and recall responses to viral infection. J. Immunol. *165*, 6081–6090.

Bousso, P., Casrouge, A., Altman, J.D., Haury, M., Kanellopoulos, J., Abastado, J.P., and Kourilsky, P. (1998). Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires. Immunity *9*, 169–178.

Brander, C., Goulder, P.J., Luzuriaga, K., Yang, O.O., Hartman, K.E., Jones, N.G., Walker, B.D., and Kalams, S.A. (1999). Persistent HIV-1-specific CTL clonal expansion despite high viral burden post in utero HIV-1 infection. J. Immunol. *162*, 4796–4800.

Busch, D.H., Pilip, I., and Pamer, E.G. (1998a). Evolution of a complex TCR repertoire during primary and recall bacterial infection. J. Exp. Med. *188*, 61–70.

Busch, D.H., Pilip, I.M., Vijh, S., and Pamer, E.G. (1998b). Coordinate regulation of complex T cell populations responding to bacterial infection. Immunity *8*, 353–362.

Chen, Z.W., Li, Y., Zeng, X., Kuroda, M.J., Schmitz, J.E., Shen, Y., Lai, X., Shen, L., and Letvin, N.L. (2001). The TCR repertoire of an immunodominant CD8$^+$ T lymphocyte population. J. Immunol. *166*, 4525–4533.

Christensen, J.P., Doherty, P.C., Branum, K.C., and Riberdy, J.M. (2000). Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of CD8+ T-cell memory. J. Virol. *74*, 11690–11696.

Cibotti, R., Cabaniols, J.P., Pannetier, C., Delarbre, C., Vergnon, I., Kanellopoulos, J.M., and Kourilsky, P. (1994). Public and private Vβ TCR repertoires against hen egg white lysozyme (HEL) in nontransgenic versus HEL transgenic mice. J. Exp. Med. *180*, 861–872.

Cose, S.C., Kelly, J.M., and Carbone, F.R. (1995). Characterization of diverse primary herpes simplex virus type 1 gB-specific cytotoxic T-cell response showing a preferential Vβ bias. J. Virol. *69*, 5849–5852.

Davis, M.M., and Chien, Y.H. (1999). T cell antigen receptors. In Fundamental Immunology, W.E. Paul, ed. (Philidelphia: Lippincott-Raven), pp. 341–366.

Doherty, P.C., and Christensen, J.P. (2000). Accessing complexity: the dynamics of virus-specific T cell responses. Annu. Rev. Immunol. *18*, 561–592.

Doherty, P.C., Allan, W., Eichelberger, M., and Carding, S.R. (1992). Roles of αβ and γδ T cell subsets in viral immunity. Annu. Rev. Immunol. *10*, 123–151.

Eichelberger, M.C., Wang, M.L., Allan, W., Webster, R.G., and Doherty, P.C. (1991). Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and CD4-depleted mice. J. Gen. Virol. *72*, 1695–1698.

Fasso, M., Anandasabapathy, N., Crawford, F., Kappler, J., Fathman, C.G., and Ridgway, W.M. (2000). TCR-mediated repertoire selection and loss of TCR Vβ diversity during the initiation of a CD4$^+$ T cell response in vivo. J. Exp. Med. *192*, 1719–1730.

Flynn, K.J., Belz, G.T., Altman, J.D., Ahmed, R., Woodland, D.L., and Doherty, P.C. (1998). Virus-specific CD8$^+$ T cells in primary and secondary influenza pneumonia. Immunity *8*, 683–691.

Flynn, K.J., Riberdy, J.M., Christensen, J.P., Altman, J.D., and Doherty, P.C. (1999). In vivo proliferation of naive and memory influenza-specific CD8$^+$ T cells. Proc. Natl. Acad. Sci. USA *96*, 8597–8602.

Garboczi, D.N., Ghosh, P., Utz, U., Fan, Q.R., Biddison, W.E., and Wiley, D.C. (1996). Structure of the complex between human TCR, viral peptide and HLA-A2. Nature *384*, 134–141.

Garcia, K.C., Degano, M., Stanfield, R.L., Brunmark, A., Jackson, M.R., Peterson, P.A., Teyton, L., and Wilson, I.A. (1996). An αβ T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. Science *274*, 209–219.

Gascoigne, N.R., Chien, Y., Becker, D.M., Kavaler, J., and Davis, M.M. (1984). Genomic organization and sequence of TCR β-chain C- and J-region genes. Nature *310*, 387–391.

Hamilton-Easton, A., and Eichelberger, M. (1995). Virus-specific antigen presentation by different subsets of cells from lung and mediastinal lymph node tissues of influenza virus-infected mice. J. Virol. *69*, 6359–6366.

Hou, S., Hyland, L., Ryan, K.W., Portner, A., and Doherty, P.C. (1994). Virus-specific CD8$^+$ T-cell memory determined by clonal burst size. Nature *369*, 652–654.

Huleatt, J.W., Pilip, I., Kerksiek, K., and Pamer, E.G. (2001). Intestinal and splenic T cell responses to enteric *Listeria monocytogenes* infection: distinct repertoires of responding CD8 T lymphocytes. J. Immunol. *166*, 4065–4073.

Islam, S.A., Hay, C.M., Hartman, K.E., He, S., Shea, A.K., Trocha, A.K., Dynan, M.J., Reshamwala, N., Buchbinder, S.P., Basgoz, N.O., and Kalams, S.A. (2001). Persistence of HIV1-specific CTL clones in a subject with rapid disease progression. J. Virol. *75*, 4907–4911.

Iwamoto, A., Ohashi, P.S., Pircher, H., Walker, C.L., Michalopoulos, E.E., Rupp, F., Hengartner, H., and Mak, T.W. (1987). TCR V gene usage in a specific cytotoxic T cell response. Primary structure of the antigen-MHC receptor of four hapten-specific cytotoxic T cell clones. J. Exp. Med. *165*, 591–600.

Jorgensen, J.L., Esser, U., Fazekas de St Groth, B., Reay, P.A., and Davis, M.M. (1992). Mapping TCR-peptide contacts by variant

peptide immunization of single-chain transgenics. Nature *355*, 224–230.

Kelly, J.M., Sterry, S.J., Cose, S., Turner, S.J., Fecondo, J., Rodda, S., Fink, P.J., and Carbone, F.R. (1993). Identification of conserved TCR CDR3 residues contacting known exposed peptide side chains from a major histocompatibility complex class I-bound determinant. Eur. J. Immunol. *23*, 3318–3326.

Kilbourne, E.D. (1969). Future influenza vaccines and the use of genetic recombinants. Bull. World Health Organ. *41*, 643–645.

Lin, M.Y., and Welsh, R.M. (1998). Stability and diversity of TCR repertoire usage during LCMV infection of mice. J. Exp. Med. *188*, 1993–2005.

Lone, Y.C., Ojcius, D.M., Bellio, M., Kourilsky, P., and Abastado, J.P. (1994). Major contribution of the β chain to the antigen specificity of a TCR. C. R. Acad. Sci. III *317*, 645–651.

Malherbe, L., Filippi, C., Julia, V., Foucras, G., Moro, M., Appel, H., Wucherpfennig, K., Guery, J.C., and Glaichenhaus, N. (2000). Selective activation and expansion of high-affinity CD4+ T cells in resistant mice upon infection with *Leishmania major*. Immunity *13*, 771–782.

Malissen, M., Minard, K., Mjolsness, S., Kronenberg, M., Goverman, J., Hunkapiller, T., Prystowsky, M.B., Yoshikai, Y., Fitch, F., Mak, T.W., et al. (1984). Mouse T cell antigen receptor: structure and organization of C and J gene segments encoding the β polypeptide. Cell *37*, 1101–1110.

Maryanski, J.L., Jongeneel, C.V., Bucher, P., Casanova, J.L., and Walker, P.R. (1996). Single-cell PCR analysis of TCR repertoires selected by antigen in vivo: a high magnitude CD8 response is comprised of very few clones. Immunity *4*, 47–55.

Marshall, D.R., Turner, S.J., Belz, G.T., Wingo, S., Andreansky, S., Sangster, M.Y., Riberdy, J.M., Liu, T., Tan, M., and Doherty, P.C. (2001). Measuring the diaspora for virus-specific CD8+ T cells. Proc. Natl. Acad. Sci. USA *98*, 6313–6318.

Maryanski, J.L., Attuil, V., Hamrouni, A., Mutin, M., Rossi, M., Aublin, A., and Bucher, P. (2001). Individuality of Ag-selected and preimmune TCR repertoires. Immunol. Res. *23*, 75–84.

McHeyzer-Williams, L.J., Panus, J.F., Mikszta, J.A., and McHeyzer-Williams, M.G. (1999). Evolution of antigen-specific TCRs in vivo: preimmune and antigen-driven selection of preferred CDR3 motifs. J. Exp. Med. *189*, 1823–1838.

McHeyzer-Williams, M.G., and Davis, M.M. (1995). Antigen-specific development of primary and memory T cells in vivo. Science *268*, 106–111.

McWilliam, A.S., Marsh, A.M., and Holt, P.G. (1997). Inflammatory infiltration of the upper airway epithelium during Sendai virus infection: involvement of epithelial dendritic cells. J. Virol. *71*, 226–236.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177–187.

Naumov, Y.N., Hogan, K.T., Naumova, E.N., Pagel, J.T., and Gorski, J. (1998). A class I MHC-restricted recall response to a viral peptide is highly polyclonal despite stringent CDR3 selection: implications for establishing memory T cell repertoires in "real-world" conditions. J. Immunol. *160*, 2842–2852.

Pannetier, C., Cochet, M., Darche, S., Casrouge, A., Zoller, M., and Kourilsky, P. (1993). The sizes of the CDR3 hypervariable regions of the murine TCRβ chains vary as a function of the recombined germ-line segments. Proc. Natl. Acad. Sci. USA *90*, 4319–4323.

Pantaleo, G., Demarest, J.F., Soudeyns, H., Graziosi, C., Denis, F., Adelsberger, J.W., Borrow, P., Saag, M.S., Shaw, G.M., Sekaly, R.P., et al. (1994). Major expansion of CD8+ T cells with a predominant Vβ usage during the primary immune response to HIV. Nature *370*, 463–467.

Rott, R., Klenk, H.D., Nagai, Y., and Tashiro, M. (1995). Influenza viruses, cell enzymes, and pathogenicity. Am. J. Respir. Crit. Care Med. *152*, S16–S19.

Saito, H., Kranz, D.M., Takagaki, Y., Hayday, A.C., Eisen, H.N., and Tonegawa, S. (1984). Complete primary structure of a heterodimeric TCR deduced from cDNA sequences. Nature *309*, 757–762.

Sourdive, D.J., Murali-Krishna, K., Altman, J.D., Zajac, A.J., Whitmire, J.K., Pannetier, C., Kourilsky, P., Evavold, B., Sette, A., and Ahmed, R. (1998). Conserved TCR repertoire in primary and memory CD8 T cell responses to an acute viral infection. J. Exp. Med. *188*, 71–82.

Stevenson, P.G., Belz, G.T., Castrucci, M.R., Altman, J.D., and Doherty, P.C. (1999). A γherpesvirus sneaks through a CD8+ T cell response primed to a lytic-phase epitope. Proc. Natl. Acad. Sci. USA *96*, 9281–9286.

Tabi, Z., Lynch, F., Ceredig, R., Allan, J.E., and Doherty, P.C. (1988). Virus-specific memory T cells are Pgp-1+ and can be selectively activated with phorbol ester and calcium ionophore. Cell. Immunol. *113*, 268–277.

Tripp, R.A., Hou, S., McMickle, A., Houston, J., and Doherty, P.C. (1995). Recruitment and proliferation of CD8+ T cells in respiratory virus infections. J. Immunol. *154*, 6013–6021.

Turner, S.J., Jameson, S.C., and Carbone, F.R. (1997). Functional mapping of the orientation for TCR recognition of an H2-Kb-restricted ovalbumin peptide suggests that the β-chain subunit can dominate the determination of peptide side chain specificity. J. Immunol. *159*, 2312–2317.

Turner, S.J., Cross, R., Xie, W., and Doherty, P.C. (2001). Concurrent naive and memory CD8+ T cell responses to an influenza A virus. J. Immunol. *167*, 2753–2758.

Usherwood, E.J., Hogg, T.L., and Woodland, D.L. (1999). Enumeration of antigen-presenting cells in mice infected with Sendai virus. J. Immunol. *162*, 3350–3355.

Walker, J.A., Sakaguchi, T., Matsuda, Y., Yoshida, T., and Kawaoka, Y. (1992). Location and character of the cellular enzyme that cleaves the hemagglutinin of a virulent avian influenza virus. Virology *190*, 278–287.

Walker, P.R., Wilson, A., Bucher, P., and Maryanski, J.L. (1996). Memory TCR repertoires analyzed long-term reflect those selected during the primary response. Int. Immunol. *8*, 1131–1138.

Wallace, M.E., Bryden, M., Cose, S.C., Coles, R.M., Schumacher, T.N., Brooks, A., and Carbone, F.R. (2000). Junctional biases in the naive TCR repertoire control the CTL response to an immunodominant determinant of HSV-1. Immunity *12*, 547–556.

Wang, F., Ono, T., Kalergis, A.M., Zhang, W., DiLorenzo, T.P., Lim, K., and Nathenson, S.G. (1998). On defining the rules for interactions between the TCR and its ligand: a critical role for a specific amino acid residue of the TCR β chain. Proc. Natl. Acad. Sci. USA *95*, 5217–5222.

Yanagi, Y., Maekawa, R., Cook, T., Kanagawa, O., and Oldstone, M.B. (1990). Restricted V-segment usage in CRs from CTLs specific for a major epitope of LCMV. J. Virol. *64*, 5919–5926.

TAB B

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Research paper

Effect of age on the repertoire of cytotoxic memory (CD8+CD45RO+) T cells in peripheral blood: The use of rearranged T cell receptor γ genes as clonal markers

Raellene Dare [1], Pamela J. Sykes, Alexander A. Morley, Michael J. Brisco *

Department of Haematology and Genetic Pathology, Flinders University and Flinders Medical Centre, Bedford Park, South Australia 5042, Australia

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Abstract

We have established a method to estimate the number of clones in peripheral blood, using rearranged T cell receptor γ genes as clonal markers, selecting cells at random, and establishing the sizes of the clones to which they belong. Clone sizes were quantified by a clone-specific PCR test based on the VNJ junctional sequence, which typically detects 1-2 copies of its target gene. All clones chosen for study were subsequently quantified in blood, and sizes ranged from 3×10^{-6} (1 cell in 330,000 CD8+CD45RO+ cells) to 3.5×10^{-2} permitting numbers of clones to be estimated from the harmonic mean of clone size. Two independent estimates from a healthy young adult (20-30 years old) gave repertoires of 94,000 and 110,000 clones. Two other healthy young adults gave repertoires of 40,000 and 55,000 clones. Repertoires in four healthy active older (>75 years old) adults were more variable but generally lower, being 3,600, 5,500, 14,000 and 97,000 clones, despite enlarged clones making up >1% of the compartment in the last individual. Overall, young adults had smaller clones ($p=0.026$, non-directional Mann-Whitney U test). If the human body contains 5 litres of blood, clones have $2 \times 10^3 - 1.0 \times 10^7$ cells in blood. These results confirm a diverse repertoire of rearranged T cell receptor gamma genes. The number of clones thus defined are broadly consistent with other estimates of repertoire, despite differences in marker genes used and subsets of cells studied.

Keywords: Repertoire development; T Lymphocytes; Memory; Human; T Cell Receptors

1. Introduction

The size of the T cell immune repertoire of a compartment is defined as the number of different clones of T cells in that compartment. Most repertoires have been estimated using T cell receptor (TCR) α and β as clonal markers, but these are only rearranged in a fraction of T cells. The TCR γ gene is rearranged in all T cells (Chhanbhai et al., 1997; Gherardi et al., 1998; Kang et al., 1998), and thus may be a more accurate marker of the entire repertoire than either TCR α or β. Like TCR α and β, the number of possible TCR γ sequences is extremely large (LeFranc, 2002) – approximately 2.5×10^{10}, but less is known about the repertoire size.

Most estimates of the TCR repertoire have been qualitative, including using spectratyping (Gorski et al., 1994; Pannetier et al., 1995; Manfras et al., 1997), denaturing gradient gel electrophoresis (Offer-

* Corresponding author. Tel.: +61 8 8204 4105; fax: +61 8 8204 5450.
E-mail address: mike.brisco@flinders.edu.au (M.J. Brisco).
[1] Current address: University of Ulster, Coleraine, Co Derry, BT52 1SA, UK.

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mans et al., 1995; Theodorou et al., 1996; thor Straten et al., 1998), single strand conformational polymorphism (Orita et al., 1989; Lynas and Howe, 1998), heteroduplex analysis (Bottaro et al., 1994; Wack et al., 1996; Martinelli et al., 1997; Shen et al., 1998) of PCR products, and the use of antibodies specific for individual V segments (Hinz et al., 1997) to examine gene expression. These methods have shown differences between a healthy control population, and a population undergoing changes in immune repertoire, such as in rheumatoid arthritis (Breedveld et al., 1995; Borgato et al., 1997), HIV infection (Rebai et al., 1994; Jason et al., 1997; Flamand et al., 1998), and ageing (Hodes, 1997). The principal differences noted have been expanded monoclonal and oligoclonal populations of T cells, which have been interpreted as indicating some changes in the sizes and possibly numbers of clones present. However, it is probably the smaller clones which contribute most of the diversity in the repertoire. Spectratyping provides no information on either clone number or on how they change if a few clones expand. On the one hand, small clones could become smaller and the total number of clones remain the same, in which case repertoire is unchanged. On the other hand, small clones could remain the same size but the total number of clones becomes smaller, in which case the repertoire is reduced. Thus measuring repertoire size requires a technique able to assess clones of all sizes, including the smallest clones.

To quantify the T cell repertoire in peripheral blood, we developed methods to assess the sizes of CD8+ CD45RO+ cell clones, and calculated repertoire from that data. This subset was chosen for initial study, because its repertoire will be important in determining T cell memory, and thus ability to respond to repeated challenges. In order to be able to study the number of clones (ie repertoire) and how that changes, it is necessary that the sizes of clones are actually measured. Thus choice of cell subset and marker are crucial.

Here, a quantitative limiting dilution PCR was used, with DNA sequences of rearranged TCR γ genes as clonal markers. These genes contain highly variable sequence at the VNJ junction (Breit et al., 1991), making them very specific markers for individual clones. PCR across the VNJ region, using primers which recognise the unique marker sequence at the junction, can detect small numbers of cells from a given clone in a blood sample. This approach has been used to detect low levels of acute lymphoblastic leukaemia in bone marrow during remission when the leukaemic clone makes up 10^{-4} to 10^{-6} of cells in the marrow, and fewer than 10 cells may be present in the samples studied (Brisco et al., 1990). The technique provides information on the size of the clones studied, and the fraction of cells which belong to clones of that size. From this information, the total number of clones (repertoire) can be calculated. We report here sizes of CD8+CD45RO+ clones in blood, determined using (a) TCR γ as a clonal marker, (b) a method to estimate the size of repertoire and (c) an investigation of how repertoire size changes over time and with age.

2. Methods

2.1. Subjects and samples

This study included 3 healthy active individuals aged 20–30 years with no evident illness or recent history of illness (Y1, male; Y2 and Y3, female), and four healthy active individuals over 75 years. Briefly, these individuals were a 77 year old female with high blood pressure (O1), an 80 year old female with previous asthma (O2), an 83 year old male with previous heart murmurs (O3), and an 83 year old male diabetic (O4). This study was approved by the Flinders Medical Centre Committee on Clinical Investigation. The subjects and quantified clones were: subject Y1, clones Y1.1–Y1.6, accession numbers AF505522–27 respectively; subject Y2, clones Y2.1–Y2.6, AF505543–48; subject Y3, clones Y3.1–Y3.5, AF505560–64; subject O1, clones O1.1–O1.6, AF505568–73; subject O2, clones O2.1–O2.5, AF505580–84; subject O3, clones O3.1–O3.3, AF505589–91; subject O4, clones O4.1–O4.4, AF505597–600.

2.2. Purification of CD8+CD45RO+ subset

2.2.1. CD8+ cell separation

Peripheral blood (40 ml) was collected in lithium heparin and transferred into two 50 ml tubes (20 ml per tube). One ml of RosetteSep™ CD8+ T cell enrichment cocktail (Stem Cell Technologies, Vancouver, BC, Canada) was added to each tube, together with 20 ml of standard phosphate buffered saline (PBS) and incubated at room temperature for 20 min with occasional mixing. RosetteSep contains antibodies to CD4, CD14, CD16, CD56, CD19 (on white blood cells) and glycophorin A (on red blood cells). Cells reacting with these antibodies on their surface become cross-linked and are pelleted during centrifugation. CD8+ lymphocytes remain in suspension and were isolated with Lymphoprep™, washed with phosphate buffered saline (PBS) containing 2% bovine serum albumin (Sigma Aldrich), and resuspended in 1 ml of the same solution.

2.2.2. CD45RO+ cell separation

A 50 μl aliquot of M–280 streptavidin Dynabeads (Dynal®, Oslo, Norway) (3.3×10^7 Dynabeads) was washed in 700 μl of PBS, then placed for 2 min at 4 °C in the Dynal Magnetic Particle Concentrator and the PBS removed. This wash step was repeated 5 further times. The Dynabeads were adjusted to 2×10^8 beads/ml. 700 μl of biotinylated anti-CD45RA antibody (PharMingen, San Diego, CA, USA; 3 ng/μl) was added to the Dynabeads which were incubated for 30 min at 4 °C and mixed occasionally. The Dynabeads were washed 5 times in PBS, 0.1% BSA to remove unbound antibody, and were re-adjusted to 2×10^8 beads/ml in PBS. An 83 μl aliquot of CD45RA+ Dynabeads was added to 300 μl of CD8+ cells with 450 μl of PBS 1% BSA, and the mixture was incubated at 4°C for 30 min. After removal of the Dynabeads, the remaining cells were washed 3 times with 700 μl of PBS, 1% BSA, and counted. The purity of the separated cells was determined using the following primary antibodies: anti-CD45RO Cy-Chrome™ (CyC); anti-CD62L Phycoerythrin (PE); anti-CD3 Biotin (Bi); and anti-CD8 fluorescein isothiocyanate (FITC); tertiary antibodies: Streptavidin PE and streptavidin-CyC (PharMingen, San Diego, CA, USA).

2.3. Separation and purity of CD8+CD45RO+ cells

At Day 0, the CD8+CD45RO+ population made up 0.8-4.8% (mean 1.9%) of total blood white cells. After purification, this subset made up 74-92% (mean 81%) of the cells present.

2.4. Large scale extraction of DNA from purified CD8+CD45RO+ cells

DNA was extracted using a Wizard® Genomic DNA purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA was resuspended in sterile water, and its concentration was estimated by applying 3 μl samples to 1% agarose plates containing ethidium bromide, and comparing their fluorescence to that of DNA standards (Sambrook et al., 1989).

2.5. Amplification and sequencing of TCR γ marker sequences

In brief, CD8+CD45RO+ T cells were limit-diluted to an average of 1-5 cell per tube in 10 μl sterile water. To release DNA, cells were heated to 95°C for 15 min, then placed at −20 °C for 1 h. Rearranged TCRγ genes were amplified in a 25 μl reaction containing 67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.45% Triton X–100, 0.2 mg/ml gelatin, 0.48 mM dATP, dCTP, dGTP (Amersham Pharmacia Biotech, Buckinghamshire, England), dUTP (ICN Biochemicals Inc., Costa Mesa, CA, USA), 1.5 mM $MgCl_2$ and 0.2 units of Taq polymerase (Fisher Biotec, Bentley, WA, Australia) using a Hybaid OmniGene thermal cycler (Hybaid, Teddington, Middlesex, UK). The PCR profile was: 92° for 5 min; then 35 cycles of: 92 °C for 45 s, 56 °C for 45 s, 72 °C for 60 s; finally, 72 °C for 5 min. Primers (Kneba et al., 1994) were a V region consensus primer (Vγ(1–8) I), and three J region primers, (Jγ1/2, JγP1/2, and JγP), used at 0.5 μM each. One μl of first round PCR product was transferred into a second round PCR. Second round conditions were the same as for the first round except that all primers were used at 0.25 μM each, the Vγ(1–8) II primer used in preference to Vγ(1–8) I, and the reaction volume was increased to 30 μl.

2.6. Sequencing of TCR γ rearrangements, and design of clone-specific primers

The PCR product was purified by electrophoresis on a 2% low melting point agarose gel, a slice containing the band of interest was cut out, and the product was extracted with a JETsorb kit (Genomed, NC, USA). The purified PCR product (at 0.3 pmoles per reaction) was sequenced in the forward direction with the VγI-seq primer (Breit et al., 1991). The PCR product was sequenced in both directions on an Applied Biosystems 371A sequencer, using DyeDeoxy terminator chemistry. In order to reverse sequence the PCR product, the M13 reverse sequencing primer (RSP) 5′ sequence (5′cag gaa aca gct atg acc3′) was added to the 5′ end of the J region sequences. Once the forward sequence had been obtained, the J region utilized in that sequence could be determined. The PCR product was then re-amplified using only one J region primer (with RSP), and purified. The PCR product was sequenced in the reverse direction with the RSP primer. V, J and N regions were identified by comparison with germline sequences. For each clone, a clone-specific primer was designed, spanning the VNJ junctional sequence. If the Jγ1 or Jγ2 region was present, this clone-specific primer was designed to be paired with a second primer in the V region; otherwise, it was designed to be paired with a second primer in the J region.

2.7. Testing clone-specific primers for sensitivity and specificity

The PCR template used for sequencing was diluted to between 5 and 10 pg/μl, then a tenfold dilution series was prepared, from 10^{-12} g/μl to 10^{-24} g/μl (1 molecule of a 200 bp PCR product is approximately 2.2×10^{-19} g). To avoid the risk of nuclease degradation of rare molecules, or their loss by absorption to plastic-ware, dilutions were carried out in 50 ng/μl DNA from peripheral blood from a healthy unrelated volunteer.

The PCR reaction included 0.25 units of uracil-DNA glycosylase (UNG) (MBI Fermentas, Vilnius, Lithuania) and 1 μg of PBL DNA from the healthy volunteer. 24 μl of PCR mix were incubated at 37 °C for 10 min, followed by 95 °C for 5 min, then placed on ice before the template was added. For each clone studied, the optimal annealing temperature between 54 to 62 °C was determined, and was used for quantitation. The PCR profile was: 92 °C for 5 min; then 60 cycles of: 92 °C for 45 s, annealing at the clone's optimum for 45 s, 72 °C for 1 min; finally, 72 °C for at least 5 min. PCR products were stored at −20 °C, and analysed by electrophoresis on 6% polyacrylamide gels.

2.8. Quantification of clones

Clones were quantified using limiting dilution PCR (Brisco et al., 1991; Sykes et al., 1992). In brief, this technique relies on an all-or-nothing endpoint, where PCR gives large amounts of product of the expected size if there were 1–2 or more target DNA molecules in the original sample, or no product of the expected size if there were no target DNA molecules in the original sample. After PCR, samples are scored as positive or negative for amplification. The average number of target molecules per tube is calculated using Poisson statistics. From this the number of clonal cells per tube was inferred. The total number of genomes added per tube was calculated from the amount of DNA, assuming 1 cell contains on average 6 pg DNA, and thus the clone was quantified as a fraction of cells studied.

Initially, 1/8th of the DNA from each sample of CD8+CD45RO+ cells was diluted 3-fold, to give between 60 ng to 600 fg DNA (10,000 to 0.1 genomes) per PCR tube, five replicate tubes at each dilution. The samples were amplified with the Vγ(1–8) II consensus primer and the Jγ1/2, JγP and JγP1/2 primers as above, products were diluted 1/100 with water and stored at −20 °C.

A 1 μl aliquot of each diluted PCR product was tested with the clone-specific primers. Limiting dilution analysis was carried out with five replicates per concentration, plus five negative controls. If the clone was not detected, an upper limit was estimated from the total amount of DNA studied.

2.9. Calculations and statistics

For each of the subjects, the mean clonal size was calculated using the harmonic mean. Repertoire (number of clones) was the inverse of this.

3. Results

3.1. TCR γ sequences

For the 7 subjects, amplifications were attempted from 376 cell samples. From these, 130 well separated PCR products were selected for sequencing; 103 sequences successfully obtained, and V and J segments identified to confirm the amplified sequences were indeed from rearranged TCR γ genes. These sequences belonged to 95 different clones. In one subject, one of the sequences (O4.3) was seen 5 times, another 4 sequences (O2.5, O4.5, O4.8, O4.13) were seen twice. Sequences for all these clones are in Genbank (accession numbers AF505522-AF505614). For 43 clones, the N regions were longer than 4 base pairs, which permitted clone-specific primer pairs to be designed (if the N region was shorter than this, primers could amplify other TCR γ rearrangements besides the target rearrangement, and specific quantification of the target clone would not be possible). Of these 43 primer sets, 8 were discarded because they were insensitive or non-specific, leaving 35 clones that were quantified.

3.2. Quantification of clone sizes

All clones for which PCR assays were designed were detected in the CD8+CD45RO+ lymphocyte samples from which the cell bearing the original marker sequence was obtained. Fig. 1 shows an example of the data used to estimate clone size. Bright bands of 124 bp, the size predicted from the sequence, are seen in the positive controls: sequencing template alone ("+"), and sequencing template with 10,000 cells from normal blood derived from an unrelated individual ("S+"). No product was seen in the five negative controls ("-ve"). The dilution series shows 5/5 PCR tests positive at 10,000 and at 3,333 genomes per PCR tube, 2/5 pos-

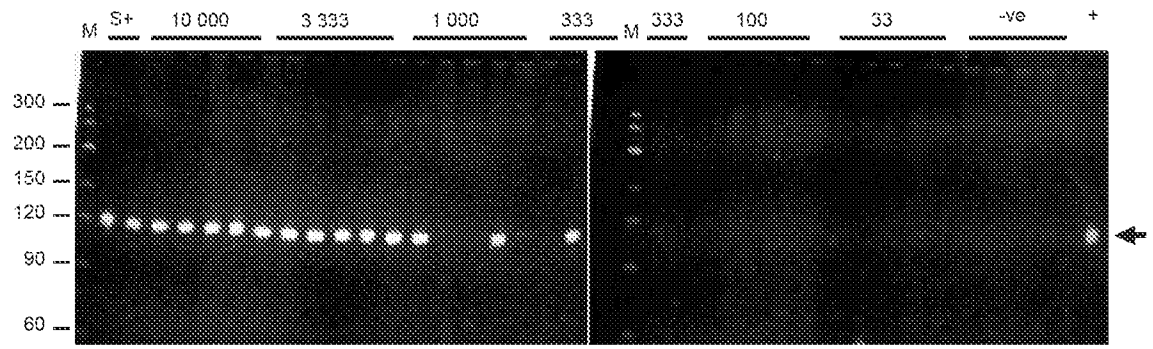

Fig. 1. Example of quantification of the size of a T cell clone, from subject Y3. Initially, a small number of CD8+CD45RO+ cells were isolated from blood. From each cell, a rearranged T cell receptor γ gene was sequenced, and a clone-specific PCR test was set up based on that marker. To measure the size of a clone, in terms of the fraction of cells it made up, a second larger sample of blood was obtained from the same person, CD8+CD45RO+ cells were again isolated, the DNA was extracted, and diluted into aliquots containing from 10,000 genomes to 33 genomes, as shown at the top of the figure. Five aliquots at each dilution, plus five negative controls with no DNA ("-ve"), were subject to a first round PCR with consensus primers, to amplify any rearranged TCR γ genes present. Next, these PCR products plus three positive controls ("+": PCR reaction containing DNA used as sequencing template; "S+": two PCR reactions each containing sequencing template plus DNA from 10,000 blood cells from an unrelated individual) were subject to a second round of PCR. This PCR used primers specific for the clone's TCR γ rearrangement. These products were analysed on a 6% polyacrylamide gel, as shown here. M: DNA size markers. Left of figure: sizes of markers, in base pairs. Right of figure: arrow indicates the clone-specific PCR product, and a size of 124 base pairs is predicted from its sequence.

itive at 1,000 genomes, 1/5 positive at 333 genomes and 0/5 positive at 100 genomes and 33 genomes. Poisson analysis of this data gives the mean frequency of this clone as 6.7×10^{-4}, or 1 clonal cell in 1,300 purified CD8+CD45RO+ cells.

Quantification of clone sizes was verified as repeatable by two independent estimates of the sizes of 6 clones from subject Y1 (Fig. 2: $r^2=0.98$). The two estimates began from two separate aliquots of genomic DNA, and involved repeating all the PCRs required for quantification of each clone.

Fig. 3 shows sizes of clones from each of the 7 subjects, expressed as fractions of the CD8+ CD45RO+ compartment, and also as clone cells per litre of blood. Size of clones within the CD8+ CD45RO+ compartment ranged over 3–4 orders of magnitude. For the young subjects, the clone sizes ranged between 3.1×10^{-6} and 3.3×10^{-3}, and for the old subjects, between 4.9×10^{-6} and 3.5×10^{-2}. When clone size was expressed as a fraction of the compartment, the differences among the young adults, and among the old adults, were not significant ($p>0.05$ for both, Kruskal-Wallis test), although, overall, young adults had smaller clones than old adults ($p=0.0257$, non-directional Mann-Whitney). When clone size was expressed as clone cells per litre of blood the differences among the young adults, and among the old adults were not significant ($p>0.05$ for both, Kruskal-Wallis test). Overall there was no significant difference between old and young adults ($p=0.1936$ non-directional Mann-Whitney).

3.3. Repertoire estimates

The most useful measure of repertoire is the number of clones, and this statistic can be calculated from data

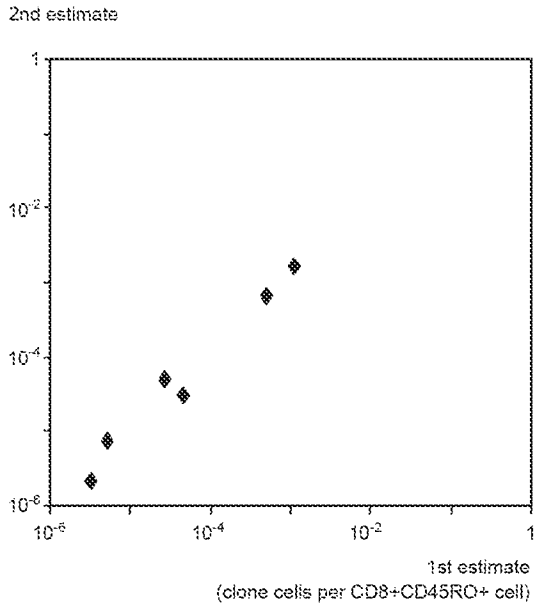

Fig. 2. Repeatability of quantifications of size of CD8+CD45RO+ clones, using the rearranged TCR γ gene as a clonal marker, and quantification with limiting dilution PCR. Sizes of 6 clones were measured independently (starting with 2 independent aliquots of genomic DNA), as a fraction of cells in the CD8+CD45RO+ compartment for subject Y1. Results were closely correlated (for log data: $Y=1.066X-0.177$; $r^2=.98$).

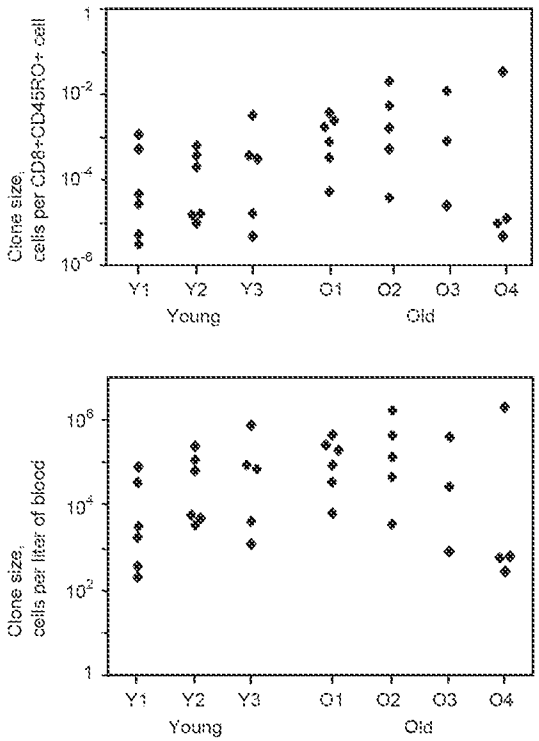

Fig. 3. Sizes of individual CD8+CD45RO+ clones from blood of three young adults (Y1 – Y3) and four older adults (O1 – O4). The top panel shows clone size as a fraction of the CD8+CD45RO+ population. The bottom panel shows clone size, as cells of the clone per litre of blood. Each point represents one clone from that donor, with up to 6 clones per donor studied.

on clone size, using the following approach. The first step is to draw up a table showing the sizes of clones found and the number of cells in the sample belonging to clones of a given size – e.g. 4 cells belong to clones of size 10^{-4}, 6 cells belonging to clones of size 10^{-5}, etc. The second step is to calculate, for each size of clone, the fraction of cells in the sample belonging to clones of that size – e.g. 40% of cells belong to clones of size 10^{-4}, 60% of cells belonging to clones of size 10^{-5}. From that we are able to infer the properties of the population of cells in blood, in terms of the fraction of cells belonging to clones of various sizes. The third step is to calculate the number of clones of each size in blood. If 100% of cells belonged to clones of 10^{-4}, there would obviously be $1/10^{-4}=10^{4}$ such clones in blood. Similarly, if 40% of cells belong to clones of 10^{-4}, blood would contain $4\times10^{-1}/10^{-4}=4{,}000$ clones of 10^{-4}. Likewise, if the other 60% of cells belong to clones of 10^{-5}, blood would contain 60,000 clones of 10^{-5}. Calculating repertoire – step four – involves adding up the total number of all clones, large and small, and in this example, repertoire would be 64,000 clones. Mathematically this is equivalent to taking the harmonic mean of clone size, and taking its reciprocal to estimate the number of clones present (e.g. for 3 clones sizes n_1, n_2, and n_3, the harmonic mean, n, is defined as follows:

$$1/n = [(1/n_1) + (1/n_2) + (1/n_3)] \times 1/3$$

Table 1 shows the repertoire of the seven subjects, based on the harmonic mean. Repertoire estimation by this method was verified as repeatable: for individual Y1, two separate estimates were made and differed by 16%. Repertoires ranged from 3.6×10^{3} clones to 9.7×10^{4} clones, with older adults tending to have smaller repertoires.

3.4. Change in size of clones over time in vivo

To examine whether repertoire changed over time, we took additional blood samples from the subjects over several months, measured clone sizes, and sought evidence that clones were changing in size or becoming extinct. Fig. 4. summarises this data. Initially clones had a median size of 37,000 cells per litre (range, 226 to 1,870,000 cells per litre), implying

Table 1
Repertoire of CD8+CD45RO+ cells in peripheral blood, in terms of numbers of clones, and means of clone size, assessed by using rearranged TCR γ genes as clonal markers

Individual	Median of clone size	Harmonic mean of clone size	Number of Clones (from harmonic mean)
Y1 (estimate 1)	3.7×10^{-5}	1.1×10^{-5}	9.4×10^{4}
Y1 (estimate 2)	4.1×10^{-5}	9.1×10^{-6}	1.1×10^{5}
Y2	1.1×10^{-4}	2.5×10^{-5}	4.0×10^{4}
Y3	3.0×10^{-4}	1.8×10^{-5}	5.5×10^{4}
O1	1.4×10^{-3}	2.8×10^{-4}	3.6×10^{3}
O2	1.6×10^{-3}	1.8×10^{-4}	5.5×10^{3}
O3	8.0×10^{-6}	7.3×10^{-5}	1.4×10^{4}
O4	1.1×10^{-5}	1.0×10^{-5}	9.7×10^{4}

Clone sizes are expressed as a fraction of cells in the CD8+CD45RO+ compartment. Averages of clone size were first estimated in terms of clonal cells as a fraction of the CD8+CD45RO+ population. Repertoire was estimated from the inverse of the harmonic mean.

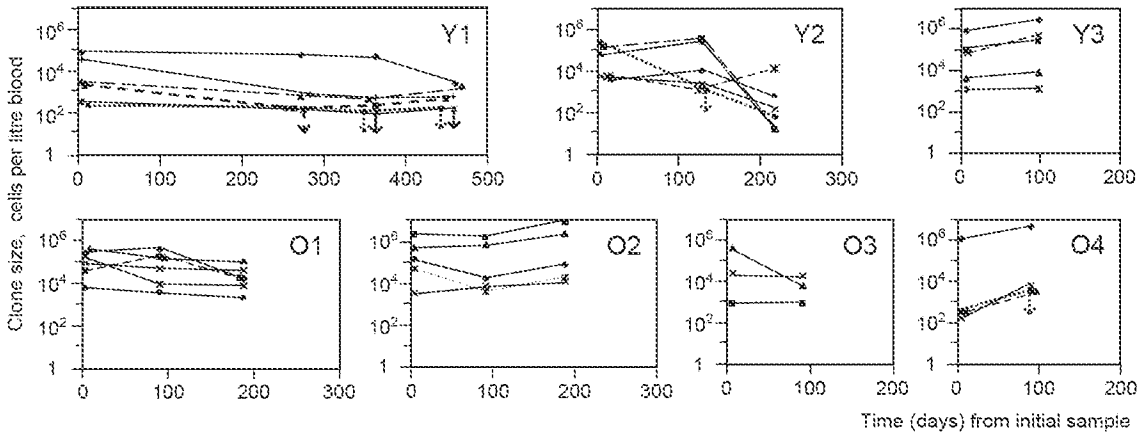

Fig. 4. Changes in sizes of CD8+CD45RO+ clones in blood of young adults (Y1 – Y3) and old adults (O1 – O4) over time. Each graph is for one subject, and dotted or dashed lines plus different symbols are used merely to identify individual clones. A down arrow indicates that the clone was not detected at that point, and thus an upper limit was estimated from the number of cells studied.

approximately 10^3 to 10^7 cells in the blood compartment (assuming 5 litres of blood/person). Most (34 of 35) clones studied were subsequently detected in at least one later blood sample. Of these, 30 clones were detected in every sample studied for that subject; two (one each in Y1 and Y2) were undetected in some samples and subsequently reappeared, suggesting some variation in size; two, both in Y1, disappeared after approximately one year, and 1 clone in O4 disappeared after a few months.

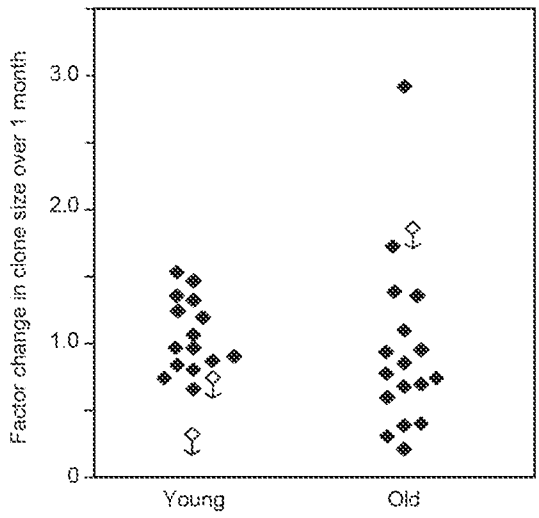

Fig. 5. Monthly rates of change in sizes of CD8+CD45RO+ clones in blood of young adults (Y1– Y3) and old adults (O1 – O4). Clone sizes were measured in terms of cells per litre, and rates of change calculated assuming clones grew or shrank exponentially. Solid symbols – clones detected in both the first and second sample. An empty symbol with an arrow – clone detected in the first sample but not the second, and thus only an upper limit for rate was estimated, from the number of cells studied.

For each time interval studied, monthly rates of clone growth or shrinkage were estimated, assuming an exponential model. Over the first time interval, for each subject, the mean net change in size per month was zero (Fig. 5) for young adults (mean 1.06; 99% CI 0.9–1.22) and old adults (mean, 0.94; 99% CI 0.58–1.30). However, 9 of the 35 clones individually showed significant changes between the first two time-points: in young adults 3 of 17 clones shrank (2 becoming temporarily undetectable) and none expanded; in old adults, 5 of 18 clones shrank, and 1 expanded. For 7 clones detectable at both time points, net changes in size per month were estimated: these ranged between shrinkage to 1/5 of the original size, and expansion to 1.7 times the original size. Of the nine clones that changed in size, seven were followed over a second time period. None of these changed further, although during that time, other clones shrank or expanded.

4. Discussion

We have quantified the sizes of CD8+CD45RO+ T cell clones in blood, using rearranged TCR γ genes as clonal markers. Within this compartment, clone sizes ranged over 3–4 orders of magnitude. Repertoires ranged from 3.6×10^3 clones to 9.7×10^4 clones, with older adults tending to have smaller repertoires. Clone sizes changed from month to month, but overall changes were slow, with most clones appearing long-lived.

This approach to calculating repertoire may seem counter-intuitive, but avoids sampling bias. As the data describe properties of a sample of clones, it ini-

tially seemed logical to draw up a frequency table showing the numbers of clones of various sizes, infer how many clones of various sizes were present in blood, and hence estimate repertoire. But the clones in the sample are a highly biased selection, mainly because a clone which is large, with perhaps 1,000 times more cells than a small clone, has a much higher chance of being picked, than a clone that is small. In other words, large clones are heavily over-represented in the sample, and the sample is very biased and unrepresentative of the situation in blood. We could not find a way to overcome this bias, either by altering experimental methods, or altering calculations. The alternative approach of using the harmonic mean is more complex but overcomes the problem. We focus not on the clones in the sample, but on the cells in the sample, and treat the data describing properties of those cells: to be precise, the sizes of clones that those cells belong to. Since all cells in blood have identical chances of being picked, the sampling is unbiased, and the properties of cells in the sample will reflect the properties of cells in blood. Proceeding in this way, it is straightforward to calculate the fractions of cells in blood that belong to clones of various sizes. Simple arithmetic then yields the numbers of clones of various sizes in blood, and the overall repertoire.

These calculations imply that methods to study repertoire must be sensitive enough to study small clones. In this study, most of the clones were small clones, and thus it is those, rather than the large clones, which will determine repertoire. This can be illustrated as follows: if equal numbers of cells belong to clones of 10^{-1} and to clones of 10^{-5}, there will be 10,000 more small clones than large clones. Thus it is quite uncertain how appearance or disappearance of large clones will affect the repertoire. On the one hand, the compartment could accommodate a large clone by shrinking other clones, and the overall repertoire might not change. In this study, the smallest clones consisted of several million cells, and thus could shrink by several orders of magnitude without becoming extinct. On the other hand, the compartment could accommodate a large clone by eliminating small clones, and the overall repertoire would be reduced. Thus the appearance of a large clone may or may not affect the body's overall ability to detect disease.

One of the subjects in this study, O4, illustrated the above point particularly well. In subject O4, 25 readable sequences were obtained which correlated to 18 different clones. One of the clones (O4.3) was seen 5 times, while 3 of the clones were seen twice. When a bulk sample of DNA was taken, TCR γ products amplified by PCR and examined by Genescan (data not shown), a prominent peak was seen, the size of which suggested that it resulted from clone O4.3. O4.3 was quantified by PCR at a level of 3.5×10^{-2}, and sequencing data suggested that it was present at 2×10^{-1} (seen in 5 of 25 cells studied), which is consistent with detection by Genescan. The most likely explanation is that this large clone was a benign expansion, such as are commonly seen in elderly people (Radl et al., 1985; Gapin et al., 1998; Silins et al., 1998). Study of other clones showed that O4 had a repertoire of 97,000 clones which is higher than average for old subjects, and certainly comparable to repertoires seen in the other old subjects who did not have expanded clones.

The estimates of the repertoire of the CD8+ CD45RO+ compartment in blood are broadly consistent with other estimates, taking into account the different cell subsets studied, and the genes used as clonal markers. Arstila et al. (1999) estimated the combined repertoire of CD4+ and CD8+ cells, using TCR β as a clonal marker, as 10^5 clones, which is slightly above our estimates of $4 \times 10^4 - 1 \times 10^5$ in young adults. Lim et al. (2002) show CD8+ frequencies which range between 2.5×10^{-3} and 4.6×10^{-6}. Wagner et al. (1998) used TCR β rearrangements to estimate the median size of CD4+CD45RO+ memory clones as around 10^{-7}, which is smaller than our estimates which ranged from 10^{-3} to 10^{-5}. The differences could be due to different behaviour of CD4+ and CD8+ cells, and to choice of different marker genes.

TCR γ and TCR β genes are rearranged at different stages in T cell development. TCR γ is rearranged first, and TCR β subsequently. Thus a single TCR γ clone could give rise to several sub-clones, each with a different TCR β rearrangement. TCR β probably provides a higher resolution marker, in that it can discriminate more clones, and this may be another factor underlying the difference between the estimate of repertoire by Wagner and co-workers, and our estimates. At this stage it is not clear whether the difference between our results, and those of Wagner et al (1998) are due mainly to the choice of cell subset, or to the choice of marker gene. We favour the former explanation, because, when Wagner and Arstila used the same marker gene to study different subsets, they came up with estimates differing by 2 orders of magnitude. This implies that different subsets might have repertoires differing markedly in size.

TCR γ can in theory discriminate a large number of clones, as the potential number of different TCR γ rearrangements is at least 2.5×10^{10} (based on 14V regions, 5 J regions and an N region of 1 to 14 base

pairs as seen here; this is a minimum estimate ignoring diversity generated by variation in V- and J-region ends). TCR β may have the disadvantage that because it is rearranged after TCR γ, some clones may be too small to detect by PCR. Wagner et al. (1998) quantified only about 50% of clones initially selected. The clones that were not detected were almost certainly the smallest clones, and if their size is unknown, the size of the repertoire will also be uncertain. In this study TCR γ was able to measure sizes of all clones encountered, including the smallest. It could be used to measure differences in repertoire between people, and also with ageing and over time. Its other advantages include the fact that it is rearranged in all T cells, whereas TCR β is rearranged only in some subsets. Moreover, it can be studied from DNA, whereas TCR β generally requires RNA studies. Using TCR β requires the use of mRNA, with even a 2–3 fold difference in transcription having the possibility of biasing the frequencies of the clones found (Maryanski et al., 1999). These practical advantages may outweigh the disadvantages of lower resolution, and TCR γ thus provides a useful method with which to study T cell repertoire.

Peripheral blood contains only 1–2% of the total lymphocyte population, raising questions about whether the memory T cells in blood are a representative sample of the immune system, or are a unique sub-set. Although different compartments of the body respond differently, and although most T lymphocytes lie outside blood, their T cell populations are linked in humans and in mice. Thus it is valid to use the blood repertoire as an index. For instance, in humans, Soares et al (2004) studied CD8+ T cells in EBV infection, in blood and tonsil tissue. In the two compartments, the clonal compositions were similar, as were cell regulation by telomere elongation, and by apoptosis. Costa et al (2003) found that in early cutaneous leishmaniasis, T cells from draining lymph nodes migrate into blood, and there are no significant differences between the surface markers of the two populations. For instance, Marshall et al. (2001) inoculated mice intra-peritoneally with one strain of influenza, challenged them with a second strain introduced intra-nasally (localizing infection to the lung) and assessed the T cell response with tetramer staining. Clones capable of responding were found in blood, marrow, spleen, liver, and lymph nodes at various sites. The strength of response differed in different tissues, but all tissues, including blood, did respond. Lin and Welsh (1998) infected mice with lymphocytic choriomeningitis virus (LCMV) and studied spectratypes of CD8+Vβ8+ cells in peripheral blood, peritoneal exudate, and spleen. In all compartments, the spectratypes were similar, suggesting that each contained similar populations of T cell clones. Selin et al. (1996) infected mice with Pichinde virus and LCMV and then used limiting dilution analysis to compare T cell populations in peripheral blood, spleen, lymph node, and peritoneal cavity. In all compartments, there were simultaneous decreases in virus-specific memory cytotoxic T lymphocyte precursors. These studies suggested that infection affected all compartments and that, afterwards memory cells in blood were not trafficking to compartments where they were protected from deletion. McCormack et al. (1993) infected mice with staphylococcus enterotoxin A, and studied T cells in peripheral blood, lymph node, spleen and mesenteric lymph node. In all compartments, there were concomitant increases and decreases in response. Thus, even though the exact relation between the blood repertoire and other repertoires is yet to be established, the studies imply that the repertoires will be linked, and suggest that blood can act as an index for the whole body repertoire. This is convenient, because in humans, blood is easy to sample, compared to the other tissues where T cells are found.

In this study, the clones changed little in size over several months, suggesting stability. This is comparable to data from Wagner et al. (1998) on CD4+CD45RO+ clones from two individuals, where they investigated 9 clones from two individuals. All clones that were initially found (6/9) were detected in blood two years later at similar levels. During our study two very small clones in individual Y1 and one in O4 became undetectable. These were already at the lower limit of detection (approximately 1 cell/ml of blood), and as clones changed only slowly in size, we presume that they simply fell below the limit of detection. The data suggests that overall, clones are long-lived, which implies that the compartment's composition is stable from month to month. Thus we found no evidence that the repertoire contracts significantly from month to month, or that clones become extinct perhaps to be replaced by others. This data, based on following individual clones with a genetic marker, is broadly consistent with immunological studies suggesting that responses can persist for long time periods. (Ahmed and Gray, 1996; Kaech and Ahmed, 2001).

However, the repertoire does shrink slightly over a human lifespan. Older adults (aged 75 or more) showed a slightly larger average clone size, and a repertoire about 1 order of magnitude lower than younger adults (aged 20-30). In older adults, repertoire sizes were more varied. This implies that in different individuals, ageing has markedly different effects on

the immune system, as noted by others (Hingorani et al., 1993; Posnett et al., 1994; Colombatti et al., 1998; Halapi et al., 1999). With ageing, antigen-driven clonal T cell expansion, as well as the decreased availability of naïve T cells, could reduce the broad diversity of the repertoire compared to that seen in early life (Schwab et al., 1997). This lower memory repertoire could reduce disease resistance in the elderly, as an elderly person may have either fewer memory clones, or no memory clones at all with which to combat a repeated challenge. Another phenomenon frequently noted with ageing is the appearance of expanded benign clones, and this was noted in at least one subject here. However, as noted above, their effect on repertoire size is, at this stage, unclear.

The findings also have implications for T cell homeostasis, since it is not only the number of T cells that matters, but their repertoire. For example, memory T cell counts stay the same throughout the lifespan apart from extreme old age (Sprent and Surh, 2002), but as we show, repertoire may differ. Recently, cytokines have been identified which can expand T cell populations and these could, perhaps, be used to treat immune dysfunction in lymphopenic individuals. In any such studies it will be important to assess T cell repertoire as well as T cell counts in order to make sure immune function is being restored.

These studies provide a means for obtaining marker genes for individual clones, and for estimating repertoire by measuring the sizes of clones that cells belong to. TCR γ may have advantages compared to other clonal markers, in that it is rearranged in all T cells, it can be studied from DNA rather than RNA, and can measure the sizes of all clones, including the smallest. The results also emphasise that different compartments in the body may have different sized repertoires. There is a clear need for controlled studies to determine the sizes of normal B- and T-cell repertoires in various compartments of the body. This first step will determine a reference range for normal repertoires in healthy people, and the next step will be to determine the role that a reduced repertoire plays in making people more susceptible to disease.

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References

Ahmed, R., Gray, D., 1996. Immunological memory and protective immunity: understanding their relation. Science 272, 54.

Arstila, T.P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., Kourilsky, P., 1999. A direct estimate of the human alphabeta T cell receptor diversity. Science 286, 958.

Borgato, L., Beri, R., Biasi, D., Testoni, R., Cugola, L., Ceru, S., De Sandre, G., Lunardi, C., 1997. Analysis of the T cell receptor repertoire in rheumatoid arthritis. Ch. Exp. Rheumatol. 15, 475.

Bottaro, M., Biondi, E.B.A., Migone, N., Crosti, L., 1994. Heteroduplex analysis of T-cell receptor gamma gene rearrangements for diagnosis and monitoring of cutaneous T-cell lymphomas. Blood 83, 3271.

Breedveld, F., Struyk, C.L., van Laar, J.M., Miltenburg, A.M.M., de Vries, R.R.P., van den Elsen, P.J., 1995. Therapeutic regulation of T cells in rheumatoid arthritis. Immunol. Rev. 144, 5.

Breit, T.M., Wolvers-Tettero, I.L.M., Hahlen, K., van Wering, E.R., van Dongen, J.J.M., 1991. Extensive junctional diversity of gamma delta T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. Leukemia 5, 1076.

Brisco, M.J., Tan, L.W., Orsborn, A.M., Morley, A.A., 1990. Development of a highly sensitive assay based on the polymerase chain reaction, for rare B-lymphocyte clones in a polyclonal population. Br. J. Haematol. 75, 163.

Brisco, M.J., Condon, J., Sykes, P.J., Neoh, S-H., Morley, A.A., 1991. Detection and quantitation of neoplastic cells in acute lymphoblastic leukaemia, by use of the polymerase chain reaction. Br. J. Haematol. 79, 211.

Chhanabhai, M., Adomat, S.A., Gascoyne, R.D., Horsman, D.E., 1997. Clinical utility of heteroduplex analysis of TCR gamma gene rearrangements in the diagnosis of T-cell lymphoproliferative disorders. Am. J. Clin. Path. 108, 295.

Colombatti, A., Doliana, R., Schiappacassi, M., Argentin, C., Tonutti, E., Feruglio, C., Sala, P., 1998. Age-related persistent clonal expansions of CD28(−) cells: phenotypic and molecular TCR analysis reveals both CD4(+) and CD4(+)CD8(+) cells with identical CDR3 sequences. Clin. Immunol. Immunopath. 89, 61.

Costa, R.P., Gollob, K.J., Machado, P.R.L., Bacellar, O.A.R., Almeida, R.P., Barral, A., Barral, A., Barral-Netto, M., Carvalho, E.M., Dutra, W.O., 2003. Adhesion molecule expression patterns indicate activation and recruitment of CD4+ T cells from the lymph node to the peripheral blood of early cutaneous leishmaniasis patients. Immunology Letters 90, 155.

Flamand, L., Crowley, R.W., Lusso, P., Colombini-Hatch, S., Margolis, D.M., Gallo, R.C., 1998. Activation of CD8+ T lymphocytes through the T cell receptor turns on CD4 gene expression: implications for HIV pathogenesis. P.N.A.S. (USA) 95, 3111.

Gapin, L., Fukui, Y., Kanellopoulos, J., Sano, T., Casrouge, A., Malier, V., Beaudoing, E., Gautheret, D., Claverie, J.M., Sasazuki, T., Kourilsky, P., 1998. Quantitative analysis of the T cell repertoire selected by a single peptide-major histocompatibility complex. J. Exp. Med. 187, 1871.

Gherardi, R.K., Farcet, J.P., Creange, A., Claudepierre, P., Malapert, D., Authier, F.J., Delfau-Larue, M.H., 1998. Dominant T-cell

clones of unknown significance in patients with idiopathic sensory neuropathies. Neurology 51, 384.
Gorski, J., Yassai, M., Zhu, X., Kissela, B., Keever, C., Flomenberg, N., 1994. Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status. J. Immunol. 152, 5109.
Halapi, E., Jeddi-Tehrani, M., Blucher, A., Andersson, R., Rossi, P., Wigzell, H., Grunewald, J., 1999. Diverse T-cell receptor CDR3 length patterns in human CD4+ and CD8+ T lymphocytes from newborns and adults. Scand. J. Immunol. 4, 149.
Hingorani, R., Choi, I.H., Akolkar, P., Gulwani-Akolkar, B., Pergolizzi, R., Silver, J., Gregersen, P.K., 1993. Clonal predominance of T cell receptors within the CD8+ CD45RO+ subset in normal human subjects. J. Immunol. 15, 5762.
Hinz, T., Wesch, F.D., Halary, F., Marx, S., Choudhary, A., Arden, B., 1997. Identification of the complete expressed human TCR V gamma repertoire by flow cytometry. Int. Immunol. 9, 1065.
Hodes, R.J., 1997. Aging and the immune system. Immunol. Rev. 160, 5.
Jason, J., Inge, K.L., Orloff, S.L., 1997. HIV antigens and T-cell receptor variable beta chain families. Scand. J. Immunol. 45, 81.
Kaech, S.M., Ahmed, R., 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. Nature. Immunol. 2, 415.
Kang, J., Coles, M., Cado, D., Raulet, D.H., 1998. The developmental fate of T cells is critically influenced by TCRγδ expression. Immunity 8, 427.
Kneba, M., Bolz, I., Linke, L., Bertram, J., Rothaupt, D., Hiddemann, W., 1994. Characterisation of clone-specific rearranged T-cell receptor gamma-chain genes in lymphomas and leukemias by the polymerase chain reaction and DNA sequencing. Blood 84, 574.
LeFranc, IMGT database: – updated 12/3/2002, the International Immunogenetics Database, http://imgt.cines.fr:8104.
Lim, A., Baron, V., Ferradini, L., Bonneville, M., Kourilsky, P., Pannetier, C., 2002. Combination of MHC-peptide multimer-based T cell sorting with the Immunoscope permits sensitive ex vivo quantification and follow-up of human CD8+ T cell immune responses. J. Immunol. Methods. 261, 177.
Lin, M.Y., Welsh, R.M., 1998. Stability and diversity of T cell receptor repertoire usage during lymphocytic choriomeningitis virus infection of mice. J. Exp. Med. 188, 1993.
Lynas, C., Howe, D., 1998. Simple, reliable detection of T cell clones by PCR-LIS-SSCP analysis of TCR gamma rearrangement. Mol. Cell. Probes 12, 41.
Manfras, B.J., Rudert, W.A., Trucco, M., Boehm, B.O., 1997. Analysis of the alpha/beta T-cell receptor repertoire by competitive and quantitative family-specific PCR with exogenous standards and high resolution fluorescence based CDR3 size imaging. J. Immunol. Methods. 210, 235.
Marshall, D.R., Turner, S.J., Belz, G.T., Wingo, S., Andreansky, S., Sangster, M.Y., Riberdy, J.M., Liu, T., Tan, H., Doherty, P.C., 2001. Measuring the diaspora for virus-specific CD8+ T cells. P.N.A.S. (USA) 98, 6313.
Martinelli, G., Farabegoli, P., Testoni, N., Terragna, C., Vittone, A., Raspadori, D., Amabile, M., Tura, S., 1997. Detection of clonality by heteroduplex analysis of amplified junctional region of T-cell receptor gamma in patients with T-cell acute lymphoblastic leukemias. Haematologica. 82, 161.
Maryanski, J.L., Autuil, V., Bucher, P., Walker, P.R., 1999. A quantitative, single-cell PCR analysis of an antigen-specific TCR repertoire selected during an in vivo CD8 response: direct evidence for a wide range of clone sizes with uniform tissue distribution. Mol. Immunol. 36, 745.
McCormack, J.E., Callahan, J.E., Kappler, J., Marrack, PC., 1993. Profound deletion of mature T cells in vivo by chronic exposure to exogenous superantigen. J. Immunol. 150, 3785.
Offermans, M.T.C., Sonneveld, R.D., Bakker, E., Deutz-Terlouw, P.P., de Geus, B., Rozing, J., 1995. Denaturing and non-denaturing gel electrophoresis as methods for the detection of junctional diversity in +rearranged T cell receptor sequences. J. Immunol. Methods 181, 101.
Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., Sekiya, T., 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. P.N.A.S. (USA) 86, 2766.
Pannetier, C., Even, J., Kourilsky, P., 1995. T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol. Today 16, 176.
Posnett, D.N., Sinha, R., Kabak, S., Russo, C., 1994. Clonal populations of T cells in normal elderly humans: the T cell equivalent to "benign monoclonal gammapathy". J. Exp. Med. 179, 609.
Radl, J., Valentijn, R.M., Haaijman, J.J., Paul, L.C., 1985. Monoclonal gammapathies in patients undergoing immunosuppressive treatment after renal transplantation. Clin. Immunol. Immunopath. 37, 98.
Rebai, N., Pantaleo, G., Demarest, J.F., Ciurli, C., Soudeyns, H., Adelsberger, J.W., Vaccarezza, M., Walker, R.E., Sekaly, R.P., Fauci, A.S., 1994. Analysis of the T-cell receptor beta-chain variable-region (V beta) repertoire in mono-zygotic twins discordant for human immunodeficiency virus: evidence for perturbations of specific V beta segments in CD4+ T cells of the virus-positive twins. P.N.A.S. (USA) 91, 1529.
Sambrook, J., Fritsch, E.F., Manatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schwab, R., Szabo, P., Manavalan, J.S., Weksler, M.E., Posnett, D.E., Pannetier, C., Kourilsky, P., Even, J., 1997. Expanded CD4+ and CD8+ T cell clones in elderly humans. J. Immunol. 158, 4493.
Selin, L.K., Vergilis, K., Welsh, R.M., Nahill, S.R., 1996. Reduction of otherwise remarkably stable virus-specific cytotoxic T lymphocyte memory by heterologous viral infections. J. Exp. Med. 183, 2489.
Shen, D.F., Doukhan, L., Kalams, S., Delwart, E., 1998. High-resolution analysis of T-cell receptor beta-chain repertoires using DNA heteroduplex tracking: generally stable, clonal CD8+ expansions in all healthy young adults. J. Immunol. Methods 215, 113.
Silins, S.L., Cross, S.M., Krauer, K.G., Moss, D.J., Schmidt, C.W., Misko, I.S., 1998. A functional link for major TCR expansions in healthy adults caused by persistent Epstein-Barr virus infection. J. Clin. Invest. 102, 1551.
Soares, M.V.D., Plunkett, F.J., Verbeke, C.S., Cook, J.E., Faint, J.M., Belaramani, L.L., Fletcher, J.M., Hammershemit, N., Rustin, M., Bergler, W., Beverley, P.C.L., Salmon, M., Akbar, A.N., 2004. Integration of apoptosis and telomere erosion in virus-specific CD8+ T cells from blood and tonsils during primary infection. Blood 103, 162.
Sprent, J., Surh, C.D., 2002. T cell memory. Annual Review of Immunology 20, 551.
Sykes, P.J., Neoh, S-H., Brisco, M.J., Hughes, E., Condon, J., Morley, A.A., 1992. Quantitation of targets for PCR by use of limiting dilution. BioTechniques 13, 444.

Theodorou, I., Bigorgne, C., Delfau, M.H., Lahet, C., Cochet, G., Vidaud, M., Raphael, M., Gaulard, P., Farcet, J.P., 1996. VJ rearrangements of the TCR gamma locus in peripheral T-cell lymphomas: analysis by polymerase chain reaction and denaturing gradient gel electrophoresis. J. Pathol. 178, 303.

thor Straten, P., Barfoed, A., Seremet, T., Saeterdal, I., Zeuthen, J., Guldberg, J.P., 1998. Detection and characterization of alpha-beta-T-cell clonality by denaturing gradient gel electrophoresis (DGGE). BioTechniques 25, 244.

Wack, A., Montagna, D., Dellabona, P., Casorati, G., 1996. An improved PCR-heteroduplex method permits high-sensitivity detection of clonal expansions in complex T cell populations. J. Immunol. Methods 196, 181.

Wagner, U.G., Koetz, E., Weyand, C.M., Goronzy, J.J., 1998. Perturbation of the T cell repertoire in rheumatoid arthritis. P.N.A.S. (USA) 95, 14447.